Pollinator-mediated evolution: a study in an orchid pollination system

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Zusammenfassung

Eines der prominentesten Merkmale der Angiospermenradiation ist die enorme Diversität von Blütenorganen, in welcher sich die mannigfaltigen Eigenschaften und Fortpflanzungsstrategien von Blütenpflanzen widerspiegeln. Die Erforschung der ökologischen und evolutionären Prozesse, welche dieser Diversität zugrunde liegen, ist deshalb nicht nur in der Blütenökologie ein zentrales Thema, sondern auch in der Evolutionsbiologie von grosser Bedeutung. Die Hypothese, dass Bestäubern eine tragende Rolle in der Beeinflussung der Evolution von Angiospermen zufällt, wurde schon von Charles Darwin formuliert und ist bis heute ein fundamentales Forschungsthema. Zwei grundsätzliche Fragen, nämlich (1) wie und unter welchen ökologischen Umständen die Blütenevolution durch bestäubervermittelte Selektion beeinflusst wird, sowie (2) in welchem Zusammenhang bestäubervermittelte Selektion und Artbildung stehen, stellen jedoch nach wie vor eine wissenschaftliche Herausforderung dar und repräsentieren kritische Wissenslücken im aktuellen Forschungsstand.

In dieser Arbeit versuche ich einige dieser Lücken im Verständnis floraler Adaptation und - Diversifikation mittels Beobachtungen und Feldexperimenten zu schliessen. Als Studiensystem dient die Nektar produzierende Orchideengattung *Gymnadenia*. Die Familie der Orchideen ist bekannt für ihre enorme Vielfalt an Formen und Bestäubungsmechanismen, und ist somit bestens geeignet für die Forschung über bestäubervermittelte Selektion und Adaptation. Die untersuchten *Gymnadenia*-Arten haben ein funktionell hochspezialisiertes Bestäubungssystem mit lang- und kurzzüngigen Schmetterlingen als Hauptbestäuber, und kommen in einem sehr grossen geographischen Bereich entlang eines ausgedehnten Höhengradienten vor.

Eine Voraussetzung für das Verständnis der Prozesse floraler Diversifikation ist die Kenntnis über die Art und Weise wie sich Selektion auf Blütenmerkmale auswirkt. In Kapitel 1 und 2 dokumentiere ich das Ausmass und die Richtung phänotypischer Selektion auf die Blütenmorphologie und -symmetrie, Blütengrösse, Farbe sowie Blütenduft in *G. odoratissima*. Die Ergebnisse zeigen regionale Unterschiede in der Selektion auf viele morphologische Merkmale wie Spornlänge und Blütenfläche, sowie auf mehrere Bestandteile des Blütenduftes. Da es zwischen

diesen Regionen Unterschiede in der lokalen Zusammensetzung der Bestäuber gibt, weisen unsere Resultate auf eine mögliche Differenz im Selektionsdruck durch diese regional verschiedenen Bestäubergemeinschaften auf Blütenmorphologie und Duft hin. Dies ist somit die erste Studie, welche konsistente regionale Selektionsunterschiede auf Blütenduft dokumentiert. Diese Studie betont ebenfalls die Wichtigkeit des Miteinbezugs räumlicher und zeitlicher Komponenten, sowie die Verwendung mehrerer Populationen in phänotypischen Selektionsstudien.

Untersuchungen floraler Evolution werden selten in einem breiten geographischen Kontext durchgeführt, und nur sehr wenige Studien zeigen lokale Adaptation als Folge von räumlicher auf räumliche Differenzierung von Bestäubern. In Kapitel 3 untersuche ich die lokale Anpassung von *G. odoratissima* auf lokale Bestäuber, indem ich (1) qualitative und quantitative Unterschiede in der Zusammensetzung der die einzelnen Pflanzen besuchenden Bestäuber dokumentiert habe, und (2) Experimente durchgeführt habe, bei denen Pflanzen reziprok zwischen geographisch und durch Höhenstufen getrennten Regionen transferiert wurden. Die Resultate weisen auf die Existenz lokaler Anpassung an Bestäuber durch Unterschiede in deren Anlockung hin. Diese lokale Anpassung wurde jedoch ausschliesslich in den Bergpopulationen ermittelt, in welchen eine vergleichsweise grössere Diversität an bestäubenden Insekten festgestellt wurde. Ich zeige hier die Wichtigkeit reziproker Transfers zur Untersuchung floraler Adaptation an ein geographisches Bestäubermosaik auf und empfehle diese Methode für solche Fragestellungen.

Um zu verstehen wie geographische Vielfalt zu Artbildung führen kann ist ein Verständnis der Evolution reproduktiver Isolation nötig. In Kapitel 4 untersuche ich die Mechanismen, welche für die reproduktive Isolation zwischen den sympatrisch vorkommenden Orchideenarten *G. odoratissima* und *G. conopsea* verantwortlich sind. Durch die Untersuchung der phylogenetischen Verwandtschaft zwischen den beiden Arten kann ich bestätigen, dass dies zwei Schwesterarten sind. Meine Ergebnisse zeigen, dass beide Arten floral komplett isoliert sind, mit keiner oder nur geringer reproduktiver Isolation nach der Bestäubung. Dies unterstützt die These, dass florale Isolation eine Schlüsselrolle in der Artbildung bei Orchideen einnimmt. Diese Studie hat zusätzlich gezeigt, dass starke florale Isolation nicht nur auf hochgradig spezialisierte Bestäubungssysteme beschränkt ist, sondern auch in einem vergleichsweise generellen System existieren kann.

Insgesamt möchte ich mit dieser Arbeit auf die fundamentale Wichtigkeit von mikroevolutionären, interspezifischen Fallstudien hinweisen, welche unser Wissen über die Rolle von Bestäubern als treibende Kraft hinter der Evolution floraler Diversität voranbringen. Die Kombination der Analyse von Bestäubern und relevanten Blütenmerkmalen zusammen mit phylogenetischen Analysen erlaubt uns, das Verständnis über die Entstehung der Diversität von Blütenpflanzen erheblich zu ergänzen und zu erweitern.

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Synopsis

Floral diversity is one of the most prominent characteristics of the angiosperm radiation, displayed in an extraordinary array of floral features and reproductive strategies. Thus, uncovering the ecological and evolutionary processes underlying this diversity continues to be a central theme in floral ecology and, indeed, evolutionary biology on the whole. The hypothesis that pollinators play a crucial role as one of the key drivers in the evolution of angiosperms dates back to Darwin, and presently still remain a fundamental topic of study. Despite this, the predominant issues of determining (1) how and under what ecological conditions pollinator-mediated selection shapes floral evolution, and (2) how pollinator-mediated selection relate to speciation, remain a challenge to address and represent a critical gap in our current knowledge.

In this thesis, I aim to address some of these gaps in our understanding of floral adaptation and diversification through field experiments and observations, employing species of the rewarding orchid genus *Gymnadenia* as a study system. The Orchidaceae family is renowned for its enormous variation in form and its diversity in pollination mechanisms, therefore rendering it ideal for studies in the context of pollinator-mediated selection and adaptation. The study species of *Gymnadenia* are functionally specialised in their pollination system, pollinated mostly by short- and long-tongued Lepidoptera. These orchid species can be located in habitats extending over a large geographical and altitudinal range.

To begin to understand floral diversification, one needs to first understand the nature of selection acting on floral traits. In Chapters 1 and 2, I documented the magnitude and direction of phenotypic selection on floral morphology, symmetry, display size, colour, and scent in *G. odoratissima*. Our major findings revealed regional differentiation in selection on many floral morphological traits, including spur length and flower area, as well as on several floral scent compounds. As there were regional differences in pollinator guilds, our results presented indications of possible differential selection imposed by these separate regional guilds on floral morphology and scent composition. This is the first study to document consistent regional differences in selection on floral scent. The study also emphasised the importance of the incorporation of spatial and temporal components in phenotypic selection studies.

Investigations of floral adaptation in a broad geographical context are rare, and fewer still are studies that demonstrate local adaptation in response to spatial pollinator differentiation. In Chapter 3, I tested for local pollinator adaptation in *G. odoratissima* by (1) assessing the qualitative and quantitative differences in composition of pollinator communities visiting the plant individuals, and (2) performing reciprocal transfer experiments between attitudinally and geographically separate regions. The results showed little to no overlap in pollinators between the lowland and mountain pollinator guilds. The existence of local pollinator adaptation through pollinator attraction was found in the mountain populations only, where pollination by a comparatively greater diversity of pollinators was also observed. I highlighted the importance and encourage the employment of reciprocal transfer experimental methods in investigating floral adaptation to geographical pollinator mosaics.

In order to understand how geographical variation can lead to speciation, one needs to understand the evolution of reproductive isolation. In Chapter 4, I explored the mechanisms responsible for the reproductive isolation between the sympatrically occurring *G. odoratissima* and *G. conopsea*. Through examination of the phylogenetic relationship between the two species, I confirmed that they have a sister-species relationship. I found complete floral isolation between the two species, with little to no post-pollination isolation, thus supporting the idea that floral isolation may have played a key role in orchid speciation. Additionally, this study indicated that strong floral isolation is not solely restricted to highly specialised pollination systems but can exist in a comparatively more generalised system.

Overall, in this thesis I aim to highlight the vital importance of these microevolutionary, intraspecific case studies in collectively developing our understanding of the role of pollinators in driving floral diversity. There is much scope for the integration of pollinator data and analyses of the relevant floral traits, in combination with phylogenetic analyses, to achieve the overarching goal of elucidating the origin of flowering plant diversity.

General Introduction

Angiosperm diversification and the role of pollinators

Angiosperms account for approximately one sixth of all species on earth (Willmer, 2011), colonising almost all habitats and exhibiting themselves as perhaps the most striking visual feature of life on the planet. The exceptional species richness and diversity has been the most outstanding characteristic that colour the angiosperm radiation. Most of the plant diversity is diagnosed by the myriad of variations in floral features such as colour, size, shape, scent, rewards, as well as pollination systems. The diversity is rendered from the elaborate female insect-mimicking *Ophrys* orchid, to the unique life cycle of the enclosed inflorescence of *Ficus*, to the largest individual flower noted for the use of distinctive rotting odour for pollination, *Rafflesia arnoldii*. Ever since the explosive origin of the angiosperms in the fossil record was recognised, it is been a central goal of plant evolutionary biology to understand the factors that promote their rapid diversification, in particular with regards to the role of floral traits and pollination systems.

Darwin (1877) considered the diversity of floral traits as 'beautiful contrivances' for the purpose of outcrossing. His insights on floral function continue to influence studies in plant evolution, with many of his initial hypotheses still being investigated in contemporary studies (as reviewed in Harder and Johnson, 2009). Since Darwin, abundant evidence have frequently attributed floral diversity and rapid speciation to floral adaptations for biotic pollination (Dodd et al., 1999, Vamosi and Vamosi, 2010, van der Niet and Johnson, 2012, Kay et al., 2006). Pollination by biotic pollinators is both more common (Renner, 1998), with approximately 80 % of plants reproducing through biotic pollination, and considerably more effective than alternative modes of abiotic pollen transfer such as via wind or water (Linder, 1998, Renner, 1998, Willmer, 2011).

Several lines of evidence indicating the significant role of biotic pollinators in angiosperm radiation include (1) the temporal alignment of the radiation of major pollinator groups and angiosperms (e.g., Grimaldi, 1999, Cardinal and Danforth, 2013, Althoff et al., 2014), (2) the 'pollination syndrome' concept where suits of floral traits are associated with the attraction of particular pollinator groups (Faegri

and van der Pijl, 1979, Fenster et al., 2004, Wilson et al., 2004, Pauw, 2006), (3) the extensive diversification in the lineages of biotic pollinators (e.g. Eriksson and Bremer, 1992, Ricklefs and Renner, 1994, Dodd et al., 1999), (4) the strong selection pressure exerted by pollinators on floral traits (e.g. Schiestl and Johnson, 2013), and (5) phylogenetic evidence of pollinator-driven diversification of plants belonging to animal-pollinated lineages (e.g. Graham and Barrett, 2004, Sargent, 2004, Kay et al., 2006, van der Niet and Johnson, 2012).

The emergence of a large body of research on the adaptive origins of angiosperm diversity through pollinator interactions have stemmed from the understanding of floral trait functions combined with knowledge on the perception and behaviour of animal pollinators (Harder and Barrett, 2006, Harder and Johnson, 2009). Increasing evidence has demonstrated that pollinators can potentially drive diversification on multiple levels of the evolutionary process. For example, micro-evolutionary studies have shown pollinator-driven phenotypic selection on floral traits through measuring fitness in correlation with natural variation in floral traits (as reviewed in Harder and Barrett, 2006, Harder and Johnson, 2009). Correspondingly, macro-evolutionary evidence from phylogenetic studies, through the increasing availability of molecular phylogenies, revealed lineage splitting is connected with pollination system shifts and correlated floral trait shifts (Whittall and Hodges, 2007, van der Niet and Johnson, 2012, Valente et al., 2012, Forest et al., 2014). The understanding of macro-evolutionary patterns is thought of as an aggregated outcome of the body of micro-evolutionary case studies on the intraspecific level. Thus, these within-species case studies in plants are highly valuable in the enhancement of our knowledge in this field.

Orchids as a study system and aims of study

In the history of evolutionary biology, orchids have played an important part in providing evidence for natural selection. Darwin was intrigued by the unique pollination mechanism of orchid pollination and elaborated on the themes of adaptation by natural selection and the advantages of cross-pollination (Darwin, 1877), laying down the ground-work for understanding floral function (as reviewed in Tremblay et al., 2005).

Figure 1. Diversity of orchids and their pollination systems. (a) *Angraecum sesquipedale*. The Long-spurred Comet orchid from Madagascar is pollinated by a long-tongue hawkmoth. This interaction is a classic example of an 'arms-race' between the orchid spur and the pollinator's tongue, (b) *Ophrys sphegodes*. This species belongs to a group of Bee Orchids known for pollination by sexual deception, through mimicry of virgin female sex pheromones as well as visual and tactile cues, (c) *Anacamptis morio*. Food deception based on the instinctive foraging behavior of pollinators is common in orchid genera such as *Anacamptis*, (d) *Bulbophyllum echinolabium*. Many *Bulbophyllum* species emit a rotting odour along with various other morphological modifications to attract fly pollinators (all photos are from http://www.natgeocreative.com).

The Orchidaceae are renowned for being one of largest and most species-rich plant families, with approximately over 2,500 species recorded to date (Govaerts et al., 2012). The question of how this large family has radiated in a comparatively short

time period to most flowering plant families, in addition to its intricate adaptations to their pollinators and elaborate pollination systems (some examples are illustrated in Figure 1), have long attracted evolutionary biologists. Thus, based on these features, orchids represent a significant model system for the investigation of pollinator adaptation. The study species we employ to address our questions are two species from the genus *Gymnadenia*; *G. conopsea* and *G. odoratissima* (Figure 2 a & b respectively).

Gymnadenia conopsea (L.) R.Br. s.l. and *G. odoratissima* (L.) Rich. are terrestrial orchids, often inhabiting calcareous soils. *G. conopsea* is found to be distributed across Eurasia, while the more sparsely distributed *G. odoratissima* is found in temperate regions of Europe. In Switzerland, the location of study, both species grow in populations scattered throughout most of the country and over a large altitudinal range, from lowland forests to subalpine meadows (Figure 2 c & d respectively). An individual consists of an inflorescence, in which flowers opening sequentially from the bottom to the top. Each flower possesses a spur that produces nectar throughout anthesis, as well as a pair of pollinaria situated above the spur entrance. The flowers produce scent attracting both diurnal and nocturnal pollinators (Vöth, 2000, Huber et al., 2005). The pollination system is considered to be functionally specialized, with pollinating insects belonging mostly to the long- and short-tongued Lepidoptera groups (Vöth, 2000, Huber et al., 2005, and references therein). On the account of three important factors (1) the intraspecies phenotypic variation, (2) the large geographic range of distribution, and (3) the functionally specialized nature of the pollination system, these orchid species are ideal for studies in understanding selection and adaptation.

The aim of this thesis is to address some of the central questions in floral diversification through a variety of experimental studies. Firstly, we aim to identify the floral signals under selection and the nature of this selection through correlation of trait variation and reproductive success, over a geographical range and over a period of two years. The floral signals under investigation are a suite of floral morphological traits, addressed in **Chapter 1**, and scent composition, colour, and display size, addressed in **Chapter 2**. As pollinator composition differs on a geographical scale, thus imposing differential selection on floral traits, there is potential for intraspecific divergent selection. This may result in geographical differentiation in floral traits. In **Chapter 3** we examine for intraspecific variation in floral traits and pollinator

ecotypes over a geographical range, and test for local pollinator adaptation to contrasting pollination environments. Local adaptation is an important component contributing to adaptive floral diversification, however, another criterion for diversification is the cessation of gene flow. We investigate in **Chapter 4** the reproductive isolating mechanism between two closely related sister species, testing for the contribution of each isolation barrier from pre-pollination to post-zygotic isolation barriers. In the following sections I expand on these above-mentioned topics and introduce their significance in the understanding of plant evolution.

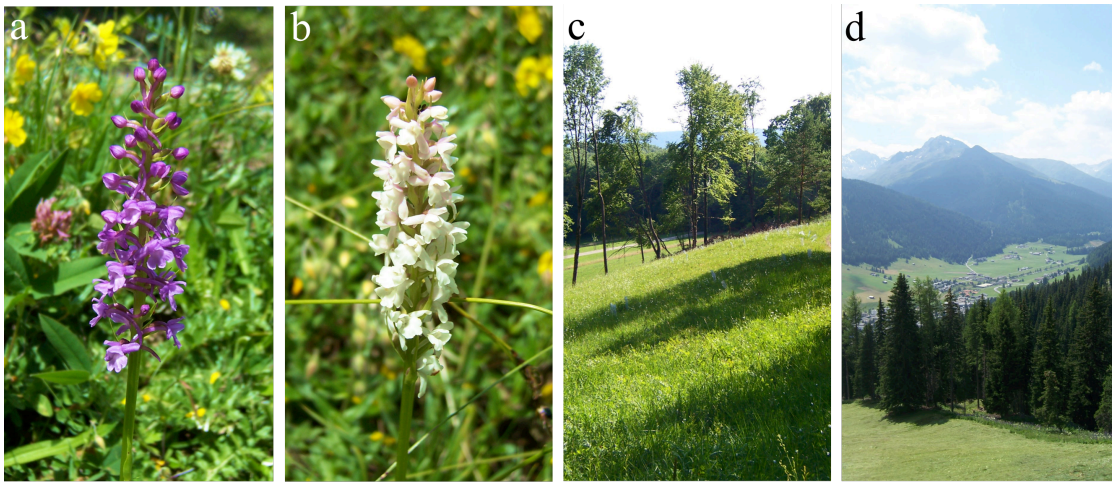

Figure 2. The study species (a) *Gymnadenia conopsea* and (b) *Gymnadenia odoratissima*, and the range of study sites in (c) the lowland, here showing the population Döttingen (500 m.a.s.l.), and (d) the mountains, here showing the population Schatzalp (1780 m.a.s.l.).

Phenotypic selection and pollinator adaptation

One of the central questions currently to address is how does selection leads to speciation? To begin with, the pathway for new species to arise by selection can be classified into two broad categories: ecological speciation and mutation-order speciation (Schluter, 2009). Here, I bring focus on ecological speciation. Ecological speciation can be defined as the development of reproductive isolation between populations or subsets of a single population by trait adaptation to different environments or ecological niches (Schluter, 2000, Schluter, 2001, Rundle and Nosil, 2005). Resurgence in the interest of ecological speciation has been observed in the last couple of decades (Schluter, 2000), following a large quantity of exploration in speciation through genetic drift (see Levin, 2000 for examples), hybridisation (e.g.

Gallez and Gottlieb, 1982, Arnold et al., 1990, Rieseberg, 1995, Rieseberg, 1997) or changes in ploidy (e.g. Soltis and Soltis, 1993). The general resulting consensus is that natural selection is the major factor in driving speciation (Rieseberg et al., 2002, Coyne and Orr, 2004, Waser and Campbell, 2004, Johnson, 2006). In the interaction between plants and pollinators, ecological speciation can be driven in allopatry or sympatry. The basic scenario of pollinator-mediated divergent selection is particular apparent in the allopatric cases, where geographically separate populations encountering different pollinators imposing selection on different floral traits and thus generating phenotypic divergence (Waser and Campbell, 2004). Factors promoting divergence among populations should remain the primary focus in the study of speciation.

Angiosperms display extensive variation in floral traits that have been established to be heritable (Ashman and Majetic, 2006), under selection (Harder and Johnson, 2009) and an important component in the process of speciation (Johnson, 2006, Kay et al., 2006). It is clear that herbivores or other antagonists, the abiotic environment, and other neutral processes can influence floral divergence (e.g. Galen, 1999, Galen, 2000, Gómez, 2003, Whittall and Strauss, 2006, Toräng et al., 2008), however changes in floral form are most frequently attributed to pollinator selection. Phenotypic selection studies in natural populations have revealed significant selection on a multitude of floral traits (as reviewed in Harder and Johnson, 2009). Some of these traits include corolla dimensions (e.g. Nattero and Cocucci, 2007, Medel et al., 2007, Anderson and Johnson, 2008, Gómez et al., 2008, Martén-Rodríguez et al., 2011, van der Niet et al., 2014), flower area (e.g. Parachnowitsch and Kessler, 2010, Sletvold et al., 2010), floral colour (e.g. Streisfeld and Kohn, 2007, Newman et al., 2012), spur length (e.g. Robertson and Wyatt, 1990, Johnson and Steiner, 1997, Anderson and Johnson, 2009, Peter and Johnson, 2014, Chapter 1), nectar properties (e.g. Johnson and Nicolson, 2008, Schlumpberger et al., 2009), floral symmetry (e.g. in zygomorphy; Moller, 1995, Rodríguez et al., 2004, Gong and Huang, 2009), and floral scent (e.g. Mant et al., 2005, Anderson and Johnson, 2009, Schiestl et al., 2011, Parachnowitsch et al., 2012, Peter and Johnson, 2014, van der Niet et al., 2014, Chapter 2).

Out of these traits, it has been suggested that in particular traits such as floral symmetry or zygomorphy and nectar spurs are features that may be involved with speciation through their effects on pollinator specificity (Kay et al., 2006). Floral

nectar spurs promote pollinator specialisation as different pollinators possess different proboscis lengths and thus may impose selection on floral spur or tube lengths. Studies have previously shown the association between the evolution of floral spurs with higher diversification in the genus *Aquilegia* (Hodges, 1997b), as well as in larger angiosperm studies (Hodges and Arnold, 1995, Hodges, 1997a). In contrast, floral symmetry, or in particular zygomorphy, can affect diversification through promoting speciation through deviation in pollinator attraction or in pollen placement on the pollinator (Johnson, 2006).

Floral scent as an important floral trait involved in selection and diversification deserves also to be highlighted, as the study of which has been largely under-represented. It is believed to be a key trait involved in plant-insect interactions (Schiestl and Johnson, 2013), however the processes leading to divergence in floral scent are currently still not well understood (Salzmann et al., 2007, Soler et al., 2011, Suinyuy et al., 2012).

Spatial-temporal components of pollinator-mediated selection

Despite the quantity of phenotypic studies, the spatial-temporal component of selection has been limited in application. Only a few studies have analysed how the direction and magnitude of pollinator-mediated selection act on intraspecific spatial-temporal floral variation with respect to geographical variation in pollinator guilds in a natural environment (as reviewed in Harder and Barrett, 2006). These particular studies are vital for the detecting reliable estimates of selection, as well as, ultimately providing the crucial link between micro- and macro-evolution of floral adaptation.

The significance of a given pollinator as a selective agent on floral traits relies on its contribution to the overall reproductive success as a medium for pollen delivery. Pollinators are thought to vary widely in their relative contribution to plant reproductive success (Herrera, 1987, Wilson and Thomson, 1991) which is defined as being proportional to the visitation rate and pollen transfer efficiency per visit (Stebbins, 1970). Divergent selection on floral traits by different pollinators can occur through adaptation to the most effective or efficient pollinator or functional pollinator group, which vary in functional morphologies, innate preferences and their abilities to perceive floral signals (Schiestl and Johnson, 2013). For most widely distributed plant species, the pollinator communities differ over a geographical scale, as well as along

altitudinal gradients. Divergence in floral morphology resulting from of divergent selection by regional differences in pollinator communities has been shown in several studies (e.g. Johnson and Steiner, 1997, Moeller, 2006, Newman et al., 2012). It was Grant and Grant (1965) who first initiated the development of a conceptual model of pollinator-driven speciation in the landmark study of the pollination of the phlox family (Polemoniaceae). A scenario was illustrated where over a spatial range, the relative abundance of two pollinators, A and B, varied. Pollinator A was the dominant pollinator of one population while pollinator B was the most frequent pollinator of another population. Thus, divergence was driven through these contrasting pollination environments, causing divergent adaptation to the respective pollinators. Stebbins later applied this theory into his 'most efficient pollinator' model, proposing that '… the characteristics of the flower will be molded by those pollinators that visit it most frequently and effectively' (Stebbins, 1970).

More recent studies have built on the Grant-Stebbins model and explored the existence and effects of pollinator mosaics in other plant genera. For example, a study in 17 plant species pollinated by a long-proboscid fly with variation in tongue length over a geographical range revealed pollinator shifts resulting from geographical divergent pollinator ecotypes across the plant habitat range (Newman et al., 2014). Another study by Nattero & Cocucci (2007) in the *Nicotiana glauca*, a hummingbird pollinated plant which exhibits geographical variation of floral traits, has shown a clear correlation between the bill length of the most frequent hummingbird pollinator out of the hummingbird assemblage with the flower corolla length.

However, there are still caveats in our understanding of pollinator-mediated geographical divergence. The demonstration of pollination ecotypes needs the determination of (1) a geographical pollinator mosaic, (2) the floral differences observed through pollinator-mediated selection, and (3) that the differences are genetically based, established through reciprocal transplantation experiments. Few studies have addressed these criteria in full (as reviewed in Herrera et al., 2006).

Pollinator ecotypes and local adaptation

One of the compelling methods for demonstrating local pollinator adaptation is through the implementation of reciprocal transplantation experiments (Ågren and Schemske, 2012, Boberg et al., 2014, Briscoe Runquist and Moeller, 2014, Sun et al.,

2014). The theory is built on the idea that if plant ecotypes are adapted to their local pollinator environment, one would expect that the ecotype would be fittest in its own local pollinator environment.

This ecotype x environment interaction response has been confirmed by a few studies employing the reciprocal transplantation experimental method. Some recent examples include the, previously introduced, guild of South-African long-tubed plants pollinated by long-proboscid flies by Anderson & Johnson (2009). In this related study, local pollinator adaptation in spur length was confirmed for a non-rewarding orchid species in the plant guild, *Disa nivea*, through reciprocal transplantation. These rarer, and non-rewarding guild members were required to 'keep up' with the changes in the adaptive race between the rewarding flowers and the pollinating flies. In another example, local adaptation in a different trait, floral colour, is demonstrated in another orchid of the same genus. Newman et al. (2012) tested the adaptive significance of flower colour in the non-rewarding *Disa ferruginea* which mimics the flower colour of sympatric rewarding species. The flowers exist in a red phenotype in the western part of the distribution range and an orange phenotype in the eastern part. Reciprocal transplantation experiments revealed ecotype x environment interaction through evidence of local colour preference by pollinators, driving adaptive floral colour shifts.

Most local pollinator adaptation studies employing reciprocal transplantation experiments have focused a singular trait, such as floral dimensions (Briscoe Runquist and Moeller, 2014), colour (Newman et al., 2012), and floral spur or tube length (Anderson and Johnson, 2009, Pauw et al., 2009, Boberg et al., 2014). The advantage of reciprocal transplantation experiments is that it can show whether there is adaptation of the overall phenotype to the local pollinator environment, however it cannot reveal the specific floral traits that have been under pollinator-mediated selection. We propose that a combination of (1) reciprocal transplantation experiments, (2) a survey of qualitative and quantitative differences in pollinator composition between communities (a simple example is shown in Figure 3 and Sun et al., 2014), (3) a survey of trait differences to include a suite of different traits, e.g. floral morphology, colour, and scent, and (4) tests for the fitness aspect of each trait in phenotypic selection experiments over a geographical region and over more than one year, would provide a stronger indication of the existence and nature of pollinator-mediated local adaptation. However, to place local pollinator adaptation in the context

of pollinator-driven speciation, a key question to address is whether pollination ecotypes can promote reproductive isolation.

Figure 3. *Gymnadenia odoratissima* from the mountain (left) and lowland region (right), with some of the major pollinators of the two regional pollinator guilds (pollinator sizes are not to scale). Mountain pollinator guild (left, from top to bottom) listed by genus unless otherwise stated: Diptera (order), Pterophoridae (family), *Eudonia*, *Zygaena*, and *Scopula*. Lowland pollinator guild (right, from top to bottom) by genus: *Oncocera*, *Phytometra*, *Ochlodes*, *Maniola*, and *Argynnis*. This diagram is adapted from data presented in Sun et al. (2014).

Floral isolation as a component of reproductive isolation

The process in which ecological divergent selection leads to reproductive isolation is a not well-established component and one of the significant challenges in empirical studies of ecological speciation (*c.f.* Nosil, 2012, Schluter, 2001). In a perhaps classic scenario, the condition for the evolution of pollinator-driven reproductive isolation is for the floral traits of plants between two pollinator communities to have diverged enough that on secondary contact both floral ecotypes would not share any

pollinators. This type of shifts in traits mediated through pollinator attraction can be thought to result in rapid floral isolation.

Floral isolation can be defined as a form of pre-pollination reproductive isolation mediated by floral morphology (morphological isolation) and pollinator behaviour (ethological isolation) (Schiestl and Schlüter, 2009). One of the most compelling examples of floral isolation is exhibited in the sexually deceptive orchids. Studies have shown that minor chemical differences in the floral scent composition are responsible for strong reproductive isolation between species (Peakall et al., 2010, Xu et al., 2011, Sedeek et al., 2014). Additionally, a recent study in another system, the Australian orchid genus *Chiloglottis*, indicated that the floral scent compounds used in pollinator attraction are the only differences that separate the three sympatric and morphologically indistinguishable taxa. These taxa are fully cross-compatible and they are described to be reproductively isolated through comprehensive nuclear and chloroplast DNA analysis (Peakall and Whitehead, 2014).

A step further in the clarification of the importance of scent in floral isolation is through experimental manipulation of the scent composition and testing for any changes to the strength of reproductive isolation barriers. We have conducted scent manipulation experiments between pairs of closely related sister-species from the sexually-deceptive orchid genus *Ophrys* and food-rewarding genus *Gymnadenia* (this data is presented in the Appendix). We aimed to reduce the difference in the scent bouquet of the species pair by separately adding the missing compounds or missing quantity of compounds to the two respective species. The pollinia were colour-stained with respect to treatment type in order to track pollen flow between the plant individuals (Figure 4A shows an example of an experimental plot, details of the methods and results can be found in the Appendix).

The results showed that for all the scent manipulation experiments in both the *Gymnadenia* and *Ophrys* genus, no breakdown of reproductive isolation (definition of terms can be found in Figure 4B) was found as pollen transfer between the scent manipulated species A individuals (‘A’ being a term of a given species) and the unmanipulated species A individuals. However, all scent manipulation experiments revealed some evidence of the breakdown of interspecies reproductive isolation, in which interspecies pollen flow between scent manipulated species A individuals and unmanipulated species B individuals have been shown (details of the results can be found in Appendix Tables A2-4). These results highlight the importance of the

specificity of the scent bouquet composition in floral isolation, in not only specialized pollination systems such as the *Ophrys* genus but also in a comparatively more generalised system of the genus *Gymnadenia*.

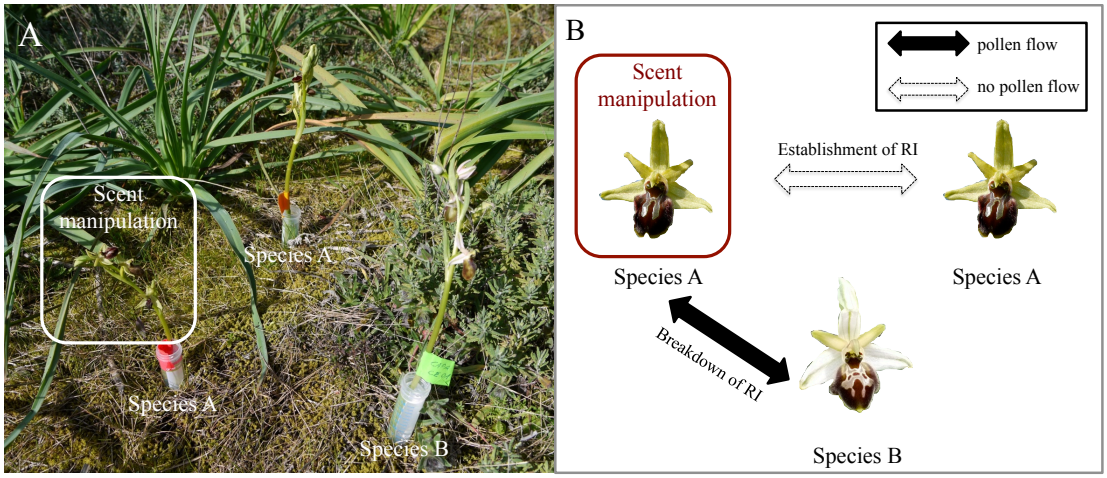

Figure 4. Scent manipulation experiment for testing the role of scent in reproductive isolation (using *Ophrys* experimental plots as an example). (A) A single manipulation plot is shown, composed of three individuals; two individuals of species A and one individual of species B. A synthetic scent blend was added to one of the two individuals of species A to reduce the interspecies scent differences. (B) Terminology of outcomes. The 'establishment of reproductive isolation (RI)' is when there is no pollen flow between the same species due to the scent manipulation, and the 'breakdown of RI' is when there is interspecies pollen flow after scent manipulation.

Within plants, multiple isolation barriers exist and have the potential to contribute to overall reproductive isolation. These barriers including pre-pollination barriers, post-pollination prezygotic barriers and post-zygotic barriers. One of the major aims in speciation biology is to determine the relative importance of different types of reproductive isolation barriers (Ramsey et al., 2003, Coyne and Orr, 2004, Nosil et al., 2005, Martin and Willis, 2007, Cozzolino and Scopece, 2008). Plant evolutionary biologists have recently made major steps towards quantifying the separate strengths of a suite of reproductive isolation barriers in several plant systems (e.g. Chari and Wilson, 2001, Ramsey et al., 2003, Husband and Sabara, 2004, Kay, 2006, Martin and Willis, 2007, Lowry et al., 2008b, Sun et al., 2015). For the most part, pre-zygotic isolation is found to be approximately twice as strong as post-zygotic isolation across many study systems, with floral isolation being the most common

form of pre-zygotic isolation barriers (Lowry et al., 2008a, Kay and Sargent, 2009, Schiestl and Schlüter, 2009).

One of the essential aspects in this area to address in the future is the quantitative determination of (1) the contribution of each individual isolation barrier to the overall reproductive isolation between closely related species, (2) the evolutionary pattern of these barriers in the speciation process, and (3) the genetic basis of these barriers (Coyne and Orr, 2004, Rieseberg and Willis, 2007, Lowry et al., 2008a, Widmer et al., 2009).

Future perspectives

In this thesis, I attempt to improve the understanding of plant speciation through the investigation of pollinator-mediated selection and adaptation, by means of the application of multidisciplinary methods. Species of the relatively widespread, food-rewarding, terrestrial orchid genus *Gymnadenia* were used to address our questions.

Since its initial concept, the model of pollinator-driven speciation developed by Grant and Grant (1965) and Stebbins (1970) has been generally supported by a wide range of studies encompassing both patterns and processes. However, certain aspects of the model still remain contentious and need further theoretical or experimental development. In particular, few studies addressing 'pollination ecotypes' have provided thorough analyses and persuasive support of the Grant-Stebbins model. More studies are necessary in understanding the processes of variation in spatial selection on floral traits in connection to geographical pollinator mosaics, particularly with the aim of adopting the five-step approach suggested by Herrera et al. (2006). The steps involve (1) unbiased sampling of geographical pollinator variation and composition, (2) testing for geographical variation in floral trait selection by pollinators, (3) examining correlation between floral trait selection and geographical pollinator variation, or 'ecotype x environment' interaction, (4) correlating pollinator selection gradients and observed phenotypic trait values, and (5) determining the existence of a genetic basis to the population differences in floral traits.

When testing for phenotypic selection, more studies measuring selection through male fitness are needed, as male and female fitness responses may not be aligned (e.g. Kulbaba and Worley, 2012) or may even be opposing (e.g. Ellis and

Johnson, 2010). Moreover, phenotypic selection studies would benefit from the use of structural equation modelling to uncover the influence of not only pollinators (e.g. Gómez et al., 2006; Chapter 1) but also a variety of other factors such as herbivores (Schemske and Horvitz, 1988, Ashman and Penet, 2007) and seed predators (Cariveau et al., 2004, Parachnowitsch and Caruso, 2008) on floral and inflorescence traits. SEM can also dissect the possible affects of trade-offs on phenotypic selection (Stanton et al., 1991, Hansen and Totland, 2006), a phenomenon that is problematic to disentangle through selection studies alone.

It is evident that under the Grant-Stebbins model, divergent pollinator adaptation and pollinator shift can result in floral isolation, and thus potentially speciation. Future studies would benefit from exploration of the importance of floral isolation barriers and the frequency in formation of these barriers in relation to other isolation barriers. Thorough analyses into the contribution of each barrier to the overall reproductive isolation, such as those illustrated by Ramsey et al. (2003), would be extremely valuable. Subsequent confirmation of gene flow barrier can be established through population genetic analysis, perhaps through use of next generation sequencing methods. Furthermore, knowledge on the selection of floral signal genes and their involvement in reproductive isolation is much needed. The molecular basis has achieved some considerable advances recently (Scopece et al., 2010), however more knowledge is needed on the role of floral signal genes in adaptive plant diversification. On the other hand, with the rise in numbers of species-level phylogenies due to an increasing abundance of new DNA sequence data, pollinator data can be integrated with ecological traits in phylogenetic analyses in identifying pollinator shifts and other ecological drivers of diversification.

With the growing change to the natural ecosystem through drastic anthropogenic influences such as climate change, habitat fragmentation and species invasion to name a few, there is increasing recognition that plant-pollinator interaction can be severely affected (e.g. Memmott et al., 2007, Hegland et al., 2009). In order to predict how these vital interactions are altered by anthropogenic changes, the underlying mechanisms need to be elucidated. This can be achieved not only through natural observational studies but also most importantly through experimental manipulation studies. These include methods mentioned in this thesis, such as (1) reciprocal transplant studies, which could be adopted to test for effects of climate

change, and (2) phenotypic trait selection studies, on native species in order to predict the spread of invasive species.

Experimental approaches to micro-evolutionary processes at the intraspecific level, such as these studies, are essential for forming hypotheses and understanding macro-evolutionary patterns. Shedding light on the ecological drivers of speciation requires a diversity of approaches from natural history to molecular biology. Ultimately, these approaches will improve our understanding of the origin of flowering plant diversity as well as future changes to our planet; undoubtedly significant goals for biologist for centuries to come.

Appendix

The following experimental data has been presented here instead of in a manuscript or chapter form due to the limited quantity of data contained at the current stage. Journal submission of this data is being further considered.

Reproductive isolation experiment in *Gymnadenia* and *Ophrys*

Method

1. *Gymnadenia*

One of the major differences in scent compound emission between the two species is the relative amount of the electrophysiologically active compound phenylacetaldehyde (PAA), emitted in high amounts by *G. odoratissima* but comparatively lower amounts in *G. conopsea* (Table A1). The scent bouquet of *G. conopsea* was manipulated through the addition of PAA to reduce the scent difference between *G. conopsea* and *G. odoratissima*. Rubber septa were soaked into this solution for two hours and then left to dry for another hour. One septum was hung on each inflorescence of *G. conopsea*, which was replaced every 48 hours. Inflorescences of *G. conopsea* and *G. odoratissima* were cut from natural populations (Döttingen, Preda and Münstertal) for plot experiments. The 'manipulation plots' contained one manipulated *G. conopsea* individual and one unmanipulated *G. conopsea* and *G. odoratissima* individual. As a control, plots without scent manipulation were set up, with each plot containing two *G. conopsea* and two *G. odoratissima*. A septum soaked in DCM was attached onto each of these individuals as a control.

The pollinia of each flower were stained in colours corresponding to their respective treatments. Over four days, the number of pollinia removed and whether massulae was received in the stigma was recorded for each flower, to determine the (1) establishment of reproductive isolation (RI), and (2) the breakdown of RI.

Table A1. Mean scent compound emission amount, range and variability in *G. odoratissima* (*n* = 20) and *G. conopsea* (*n* = 20).

	PAA (ng/l)	Total scent bouquet amount (ng/l)
Mean (± SD) absolute amounts in floral emission in *G. odoratissima*	119.01 ± 133.94	788.31 ± 586.94
Min-max absolute amounts in floral emission in *G. odoratissima*	6.61 - 401.77	
Mean (± SD) absolute amounts in floral emission in *G. conopsea*	13.88 ± 13.53	1242.40 ± 2508.50
Min-max absolute amounts in floral emission in *G. conopsea*	3.13 - 49.98	

2. *Ophrys*

For the two *Ophrys* species, *O. sphegodes* and *O. exaltata*, it has been established that the scent compounds of *O. sphegodes* are composed primarily of (Z)-9-alkenes and (Z)-12-alkenes, while *O. exaltata* has mostly (Z)-7-alkenes (Schlüter et al., 2011, Xu et al., 2012, Sedeek et al., 2014). The scent manipulation of the two species is achieved the mixing of a blend of synthetic (Z)-7-alkenes and (Z)-9-alkenes ((Z)-12-alkenes were not include in the mix to simplify the process of producing scent blends) in the observed ratios, and applied onto the labellum of *O. sphegodes* and *O. exaltata* respectively to decrease their scent differences. The experiment was performed in two populations, Marina di Lesina and Capoiale, in the region of Gargano, South Italy. In Marina di Lesina four series of plots and in Capoiale three series of plots were performed, with 10 manipulation and 10 control plots. The manipulation plots consist of two plants of ‘species 1’ of which one had the compound blend of ‘species 2’ applied and one plant of ‘species 2’, and vice versa. The control plots consist of two plants of ‘species 1’ and one plant of ‘species 2’, and vice versa.

The pollinia of each flower were stained in colours corresponding to their respective treatments, enabling the tracking of pollinator movements between the plants. The number of pollinia removed and if coloured-stained massulae was received in the stigma was recorded for each flower.

Results

Table A2. The number of stained pollen transfer events occurring between *Gymnadenia* individuals within (A) scent manipulated plots, and (B) control plots. Of the 105 individuals stained for this experiment, a total of 45 individuals were used the manipulation plots and 60 individuals were used in control plots. Within these individuals, a total of 102 stained pollination events occurred.

A	Manipulated *G. conopsea*	Control *G. conopsea*	Control *G. odoratissima*
Manipulated *G. conopsea*	32		
Control *G. conopsea*	9	10	
Control *G. odoratissima*	1	0	12

B	Control *G. conopsea*	Control *G. odoratissima*
Control *G. conopsea*	32	
Control *G. odoratissima*	0	16

Table A3. The number of stained pollen transfer events occurring between manipulated *Ophrys sphegodes* and unmanipulated *O. exaltata* individuals within (A) scent manipulated plots, and (B) control plots. A total of 240 individuals were used the manipulation plots and 150 individuals were used in control plots. Within these individuals, a total of 75 stained pollination events occurred.

A	Control *O. sphegodes*	Manipulated *O. sphegodes*	Control *O. exaltata*
Control *O. sphegodes*	10		
Manipulated *O. sphegodes*	5	11	
Control *O. exaltata*	0	3	28

B	Control *O. sphegodes*	Control *O. exaltata*
Control *O. sphegodes*	15	
Control *O. exaltata*	0	3

Table A4. The number of stained pollen transfer events occurring between manipulated *Ophrys exaltata* and unmanipulated *O. sphegodes* individuals within (A) scent manipulated plots, and (B) control plots. A total of 150 individuals were used the manipulation plots and 150 individuals were used in control plots. Within these individuals, a total of 139 stained pollination events occurred.

A	Control *O. sphegodes*	Manipulated *O. exaltata*	Control *O. exaltata*
Control *O. sphegodes*	4	1	0
Manipulated *O. exaltata*		35	33
Control *O. exaltata*			24

B	Control *O. sphegodes*	Control *O. exaltata*
Control *O. sphegodes*	13	0
Control *O. exaltata*		29

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Chapter 1.

REGIONAL DIFFERENCES IN SELECTION ON FLORAL MORPHOLOGY

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Author contributions
MS, KG and FPS designed the experiment
MS and KG performed the field experiments
MS performed the data analysis, KG provided advice on statistical methods
MS wrote the paper, contributions were made by FPS and KG

Regional differences in selection on floral morphology

Mimi Sun, Karin Gross, and Florian P. Schiestl

Abstract

Pollinators have been shown to be one of the major selective forces on floral traits, shaping the evolution of flower phenotype. Pollinator-mediated selection on pollination-related traits is well documented, however, divergent selection as a source of local or regional trait adaptation to spatial differences in pollinators received comparatively less attention. We document the direction and magnitude of phenotypic selection on floral morphology and symmetry in the food-rewarding orchid *Gymnadenia odoratissima* in several lowland and mountain populations over two years. The selection analyses showed significant differences in the floral traits that were under selection between the lowland and mountain regions. Six out of nine morphological traits measured in this analyse were under significantly stronger positive selection in the lowland region compared to the mountain region. Structural equation modeling indicated that spur length was under positive selection in the lowlands only, while most other traits were under positive or negative selection in the mountains. Floral symmetry was not found to be under selection in both regions. In addition to previous studies showing regional differences in pollination guilds, our results presenting floral morphological separation between regions bring together indications of possible differential selection by separate regional pollinator guilds.

Keywords: floral evolution, phenotypic selection, selection gradients, structural equation model, floral traits, pollinators, floral spur, floral symmetry, Orchidaceae, *Gymnadenia odoratissima*

Introduction

The main theory put forth to explain the diversity of flowering plants centres around the concept of pollinator-driven specialisation generating rapid floral evolution (Herrera et al., 2006, Fenster et al., 2004, Johnson, 2006, Harder and Johnson, 2009, Schiestl and Schlüter, 2009, and references therein). For angiosperms, the attraction of pollinators and ensuring effective pollen transfer are the primary targets of selection in the course of floral evolution, which leads to the some of the major diversification of floral forms. Understanding the shaping of individual floral traits offers valuable insights into the processes that drive the evolution of flower morphology in its entirety. In particular, exploring the pollination systems and the patterns of correlated trait evolution would considerably contribute to our understanding of the processes that has led to the variation of floral morphology observed (Pérez et al., 2006).

One of the most crucial aspects of study in the selection on floral traits involves the understanding of spatial and temporal variation in selection (Harder and Barrett, 2006). Many previous studies have evaluated pollinator-driven phenotypic selection on floral traits within a population by assessing fitness outcomes of natural floral variation (e.g. Campbell et al., 1991, Herrera, 1993, Maad, 2000) or artificially altered floral variation (e.g. Herrera, 2001, Castellanos et al., 2004, Aigner, 2004). However, there is a lack of empirical evidence in floral diversification and adaptation that examines intraspecific floral variation over a geographic area and over more than one year. Divergence in floral morphologies resulting from the use of different subsets of pollinator groups can give insights into the processes that lead to morphological shifts over time (Grant and Grant, 1968, Armbruster and Webster, 1982, Armbruster et al., 1994, Johnson and Steiner, 1997, Hansen et al., 2000, Fenster et al., 2004, and references therein).

The assessment of pollinator-driven floral morphological selection requires taking into account the morphological traits that are involved in pollinator attraction or fit. Numerous studies have shown pollinator differential preferences in flower exhibiting different size and shape, in particular, these traits include flower corolla shape (e.g. Gomez et al., 2008) and length (e.g. Nilsson, 1988, Johnson and Steiner, 1997, Anderson and Johnson, 2008), nectar spur length (e.g. Whittall and Hodges, 2007, Boberg et al., 2014), and flower area (e.g. Parachnowitsch and Kessler, 2010,

Sletvold et al., 2010). Research into floral symmetry has also received considerable attention in the past years, in particular zygomorphy which is thought to be resulting from strong pollinator selection (Neal et al., 1998, Endress, 2001). Zygomorphy is found to increase the attractiveness of the flowers (e.g. Møller, 1995, Rodríguez et al., 2004, Gong and Huang, 2009) and the pollen-transfer efficiency (Endress, 1999).

The Orchidaceae are among the most species-rich plant families and renowned for vast ecological and spectacular floral diversity and their close intricate associations with pollinators. These factors render orchids an ideal model for evolutionary studies, though few examples of natural selection in orchids exist currently (e.g. Tremblay et al., 2005, Schiestl and Schlueter, 2009, Schiestl et al., 2011, Schiestl, 2012). In this study, we aim to address gaps in our knowledge of spatial and temporal floral variation through the assessment of selection on floral traits of the food-rewarding orchid *Gymnadenia odoratissima* in multiple populations and over two years. This orchid species of study occurs over a large distribution range and over an altitudinal gradient. Previous studies in this species have shown that the pollinator guilds differ between altitudinal regions and populations in this species, with some evidence of local pollinator adaptation (Sun et al., 2014). Given our understanding so far, we explore possible spatial differentiation in selection of floral morphology through addressing the following questions: (1) Are floral morphological traits under selection, and if so, which traits? (2) Does selection on morphological floral traits differ between populations and between altitudinal regions? (3) Is floral symmetry under selection?

Material and methods

Study system

Gymnadenia odoratissima (L.) L.C.M. Richard (Orchidaceae) is an European terrestrial orchid species found in temperate and mountainous habitats. The species inhabit calcareous dry and damp substrates, and can be found in altitudes of up to 2600 m a.s.l. The species has a flowering period generally from June to August. Flowering occurs acropetally within a single inflorescence, which is composed of between 10-100 flowers. The species is food-rewarding by means of producing nectar contained in a floral spur. Above the spur entrance is a column holding two pollinaria

comprised of numerous massulae (packages of pollen; Nazarov and Gerlach, 1997). The labellum is pronounced and features three lobes; the largest one being the middle lobe (termed 'major lobe' from here on). Pollination is achieved by recruitment of diurnal and nocturnal, long- and short-tongue Lepidoptera species (Vöth, 2000, and references therein, Huber et al., 2005, Sun et al., 2014).

Study sites and fruit set data collection

The study was performed in seven populations, three lowland and four mountain populations, in Switzerland over a period of two years (see Table S1 for details of the populations and sample sizes). Within each population, 100 individuals were randomly selected and marked with tags during the anthesis stage. Subsequently, two open flowers per individual were removed with small scissors; one open flower from the top and one from the bottom of the inflorescence. These flowers were stored in 5.0 ml Eppendorf Tubes® (Eppendorf AG, Hamburg, Germany) containing 70 % ethanol.

After fruit capsule maturation, the number of fruit capsules formed per individual was recorded for all tagged individuals. Due to plant damage and missing labels, the fruit set of some of the originally tagged individuals could not be quantified. The relative female reproductive success was calculated as number of fruits of an individual divided by the population mean of the number of fruits of all individuals within the same population.

Floral morphology measurements

In total, 1722 flowers from 861 individuals of *G. odoratissima* from 2010 and 2011 were used for measurements. In the lab, each individual flower was placed in a clear petri dish, and thinly immersed in two to three drops of 70 % ethanol. The flowers were carefully spread out using a pair of fine tweezers in order to make all dimensions of the floral features fully visible. The flowers were displayed facing down such that the spur was entirely visible, revealing the exact location of the spur entrance, and then flattened into this position. Individual flower photos were taken using a digital SLR camera (Nikon D90 D-SLR; Nikon Corporation, Japan) fitted with a 105 mm F/2.8D lens (AF-S VR Micro-Nikkor; Nikon Corporation) and attached onto a fixed tripod.

For floral trait measurements, the photos were uploaded onto the image-processing and analysis program ImageJ 1.47 (NIH Image, Bethesda, Maryland, USA, http://rsbweb.nih.gov/ij/). For each flower, nine traits were measured to the nearest 0.001 mm with each measurement calibrated to a 5 cm scale included in each photo (Figure 1), while the 'floral area' trait was derived. The measurements for analysis of floral symmetry are shown in Figure 2, used to derive the degree of symmetry of the outer sepals, labellum and the major lobe of the labellum. For each trait, the mean value was obtained by averaging each trait measurement between the two flowers of each *G. odoratissima* individual.

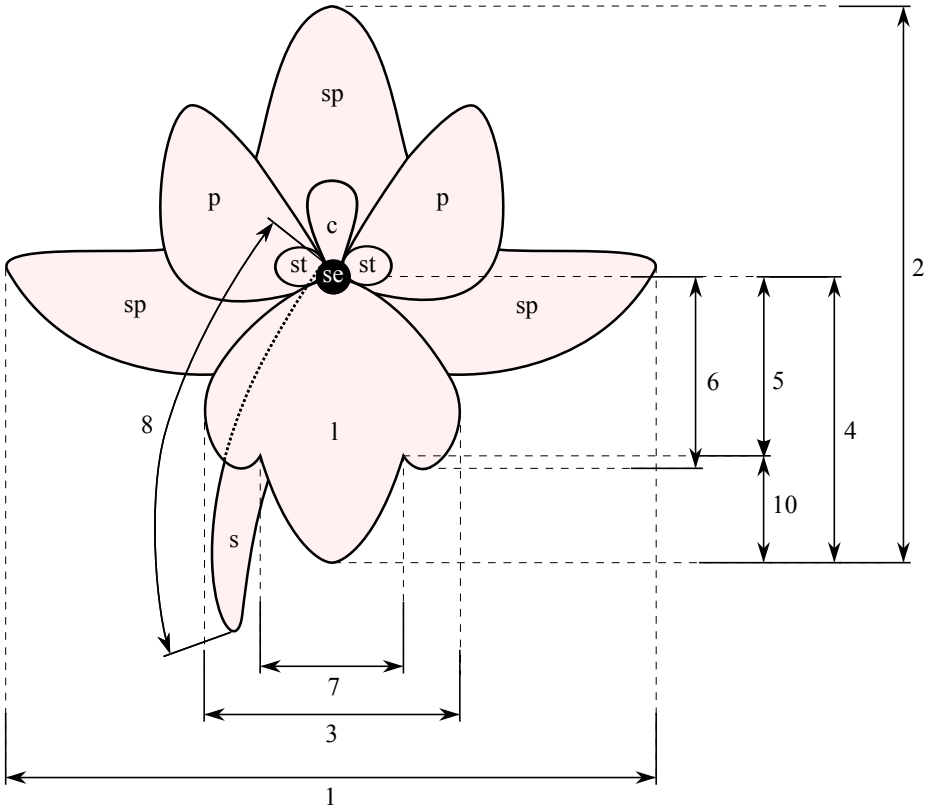


Figure 1. A diagrammatic *Gymnadenia odoratissima* flower with morphological traits 1–8 and 10 indicated. The flower morphological traits are flower width (1), flower height (2), labellum width (3), labellum height (4), spur entrance to height of interlobe (5), side-lobe length (6), interlobe distance (7), spur length (8), flower area (9, calculated by ImageJ from manually tracing the flower outline), lobe length (10). Other floral features indicated are sepals (sp), petals (p), spur (s), spur entrance (se), stigma (st), column (c), labellum (l).

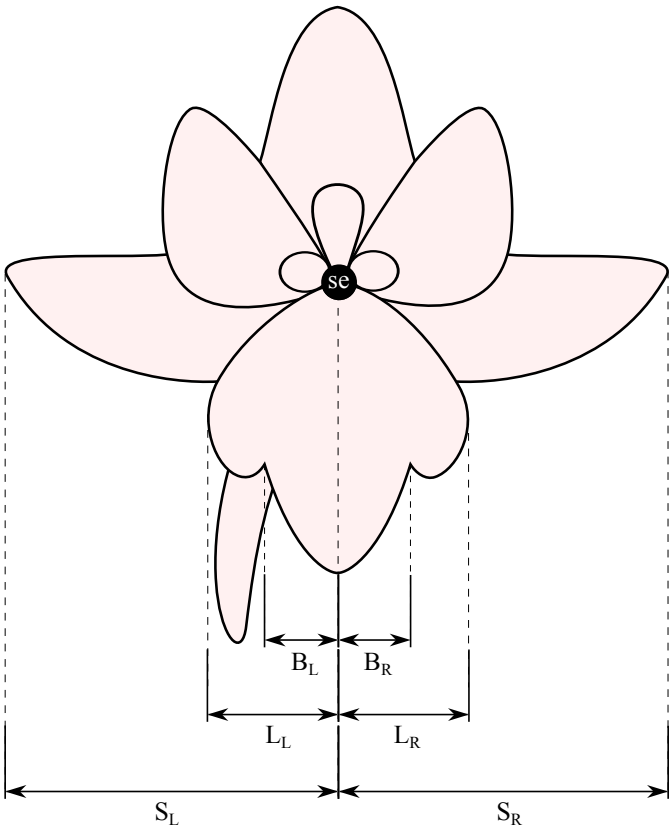


Figure 2. Morphology measurements used for floral symmetry analysis indicated on a diagrammatic *Gymnadenia odoratissima* flower. Refer to Figure 1 for floral features. The morphology measurements are left sepal (S_L), right sepal (S_R), width of the left side of labellum (L_L), width of right side of labellum (L_R), width of left side of the major lobe (B_L), width of the right side of the major lobe (L_R).

Statistical analysis

The computer software R (version 2.13.0, http://www.r-project.org/.) was used for all statistical analyses.

Morphological selection analysis

A principal component analysis (PCA) was conducted for the 10 morphological traits (Figure 1) to reduce the potentially correlated variables into principal components (PCs). The PCAs were conducted using trait values standardized (mean = 0, SD = 1, within populations) and varimax rotation. PCs with an eigenvalue greater than 1 were extracted and used as explanatory variables in the analyses. Selection gradients β were estimated through linear multiple regression analysis using relative female

reproductive success as a response variable and PCs as explanatory variables. To test for possible differences in selection between the altitudinal regions and the two years, linear mixed models with population as a random factor were used with the R package *lme4* (Bates et al., 2014). To test for differences in selection among populations within altitudinal regions and between years, linear models were conducted.

Structural Equation Model

We used structural equation modeling with latent constructs (SEM; Bollen, 1989, Shipley, 2002, Pugesek, 2003) with the aim of estimating the relationship between the measured morphological traits and reproductive success in the two altitudinal regions. The benefits of this method are that it allows the visualization of flower morphology as a single, inclusive, and multidimensional character, as well as allowing for the examination of complex direct and indirect relationships among variables. A latent variable was constructed to include the majority of morphological traits, while spur length and flower area were included as separate indicator variables of particular interest. Due to the large variance in estimates for flower area, the area measurements were log-transformed. Initially, two models (Figure S1) were constructed based on the trait grouping from principle component factors (Table 2), but alternations to the model were implemented for a better model fit.

The process of fitting structural equation models requires the testing of several models through the modifications of the initial model. Modification indices were used as a guide for minor model modifications to improve fit. Model adjustments were implemented only if they are suitable on a theoretical basis. Separate models were constructed for the lowland and the mountain region. The structural equation modeling was performed with the R package *lavaan* (version 0.5-17; Rosseel, 2012).

Differentiation of morphology between regions and populations

As it was not possible to exclude that flowers from the same individual could have been measured during both years, data from only one year (2011) were analysed. To test for differences in morphological traits between altitudinal regions and populations, we performed a generalised linear model with altitudinal region as well as population nested within region as explanatory variables.

Floral symmetry analysis

To test whether there is a significant difference in symmetry of the traits petals, labellum and major lobe of flowers occurring in different altitudinal regions and populations, generalised linear models were performed with each symmetry trait ratio as the response variable and 'altitudinal region' and 'population' nested within region as explanatory variables. The symmetry ratio of each of the three traits was calculated as follows:

$$\text{Trait symmetry ratio} = 1 - \frac{Side\text{min}}{Side\text{max}}$$

The term '*Side*min' means the smaller measurement out of the left or right component of the trait, and '*Side*max' means the larger measurement. Thus, a symmetry ratio of 0 suggests complete symmetry while ratios >0 suggest deviation away from complete symmetry.

Linear multiple regression analysis was employed to estimate selection gradients, using relative female reproductive success and the symmetry trait ratios. To test for differences in selection between altitudinal regions, linear mixed models were conducted. To test for differences in selection among populations within each altitudinal region, linear models were conducted.

Results

Phenotypic selection on morphological floral traits

The PCA produced two PCs; PC1 contained six out of the nine morphology traits used in the analysis, and PC2 contained most of the labellum traits or major lobe traits (Figure 3B and Table S2). On the regional level, there was directional selection on PCs in both altitudinal regions (Figure 3A). PC1 was under significant positive selection in the lowland region only, while PC2 was significantly selected for in the mountain region only. There were no differences in directional selection between the years for both PCs, however differences in selection were found between regions (Table 1). Flowers of the in the mountain region were under stronger positive selection for PC2 compared to that of the lowland region, however this difference was not found to be significant.

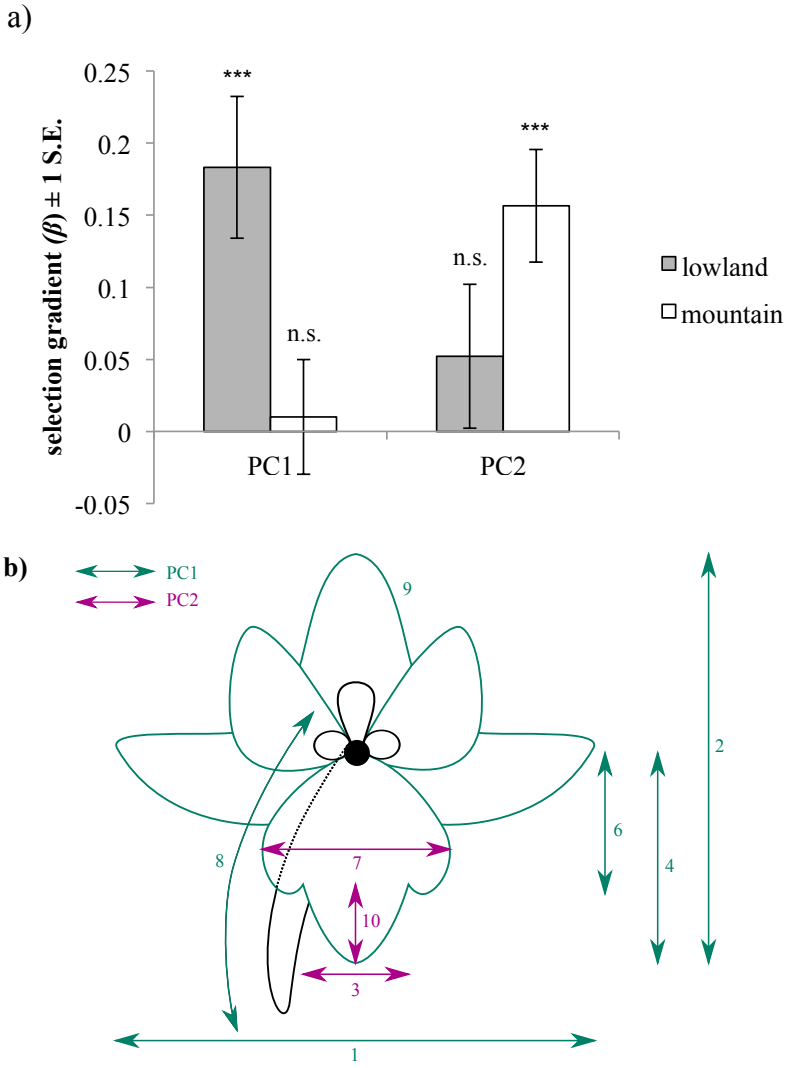


Figure 3. (a) Linear selection (β) ± 1 SE gradient differences for the two principal components (PCs) of the lowland and the mountain altitudinal region in *Gymnadenia odoratissima*. Data from three lowland populations (Doettingen, Linn, Remigen, $N_{lowland}$ = 481) and four mountain populations (Albulapass, Corviglia, Muenstertal, Schatzalp, $N_{mountain}$ = 472) in the years 2010 and 2011 were used in the analysis. Significance in directional selection for each PC in each region are indicated above each bar, denoted as "***" = $P < 0.001$, "**" = $P < 0.01$, "*" = $P < 0.05$. (b) The major loadings of the morphological trait from 1-10 on the two principal components (refer to Figure 1 as reference on the indicated traits). Trait nine indicates flower area.

Table 1. Differences in directional selection on the principle components (PCs) between altitudinal regions and between years, analysed through linear mixed models. Significant interactions are highlighted in bold.

PC	PC x region		PC x year	
	χ^2_1	*p*	χ^2_1	*p*
PC1	7.235	**0.007**	0.588	0.588
PC2	2.741	0.098	2.370	0.124

On the population level, there was significant directional selection in both altitudinal regions and years for both PCs (Figure S2). PC1 was under significant positive selection in some of the lowland and mountain populations, while PC2 was under significant positive selection in mountain populations only. There were significant differences in directional selection on PC1 in both altitudinal regions between the two years (Table 2). However, no differences in directional selection have been found for both PCs between the populations of the lowland and also between the populations the mountains.

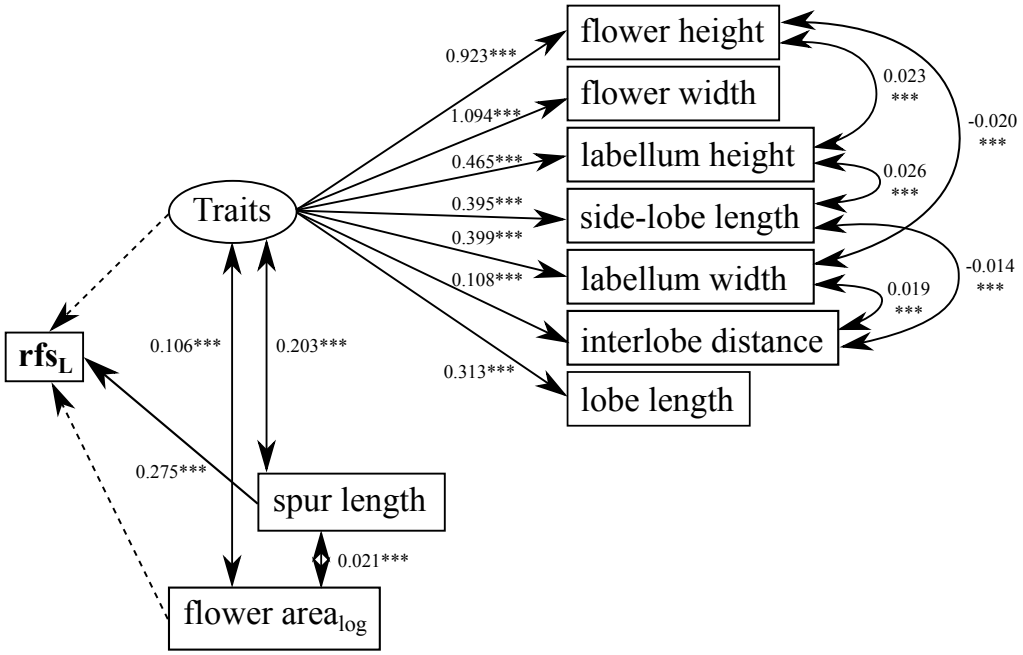
The best fitting SEM models for the two altitudinal regions are shown in Figure 4, with model fit results summarised in Table S3. Although the overall fit of the models are not completely satisfying from the results, this may be due to various factors. It may be that constructing a latent variable 'Traits' composed of several well-defined trait measurements is redundant. However, as the trait indicators under 'Traits' are highly correlated, placing them under a latent construct would be appropriate.

There is significant selection detected for spur length in the lowlands only, while there is significant selection for flower area and morphological traits under the variable 'Traits' in the mountains only. The latent variable 'Traits' is significantly correlated with the seven morphological trait indicators, aside from 'lobe length' in the mountains. Additionally, 'Traits' is negatively correlated with the reproductive success in the mountain plants. 'Traits', spur length and flower area co-vary significantly, in addition to covariation within many trait indicators under the 'Traits' variable (Figure 4) in both altitudinal regions.

Table 2. Differences in directional selection on the principle components (PCs) among populations and between years separately for the lowland region and mountain region, using linear models. Significant interactions are highlighted in bold.

PC	lowland				mountain			
	PC x population		PC x year		PC x population		PC x year	
	F_3	p	F_1	p	F_2	p	F_1	p
PC1	1.620	0.447	6.037	**0.015**	2.595	0.145	3.853	**0.005**
PC2	2.188	0.337	0.670	0.414	2.736	0.128	0.045	0.760

A) Lowland



B) Mountain

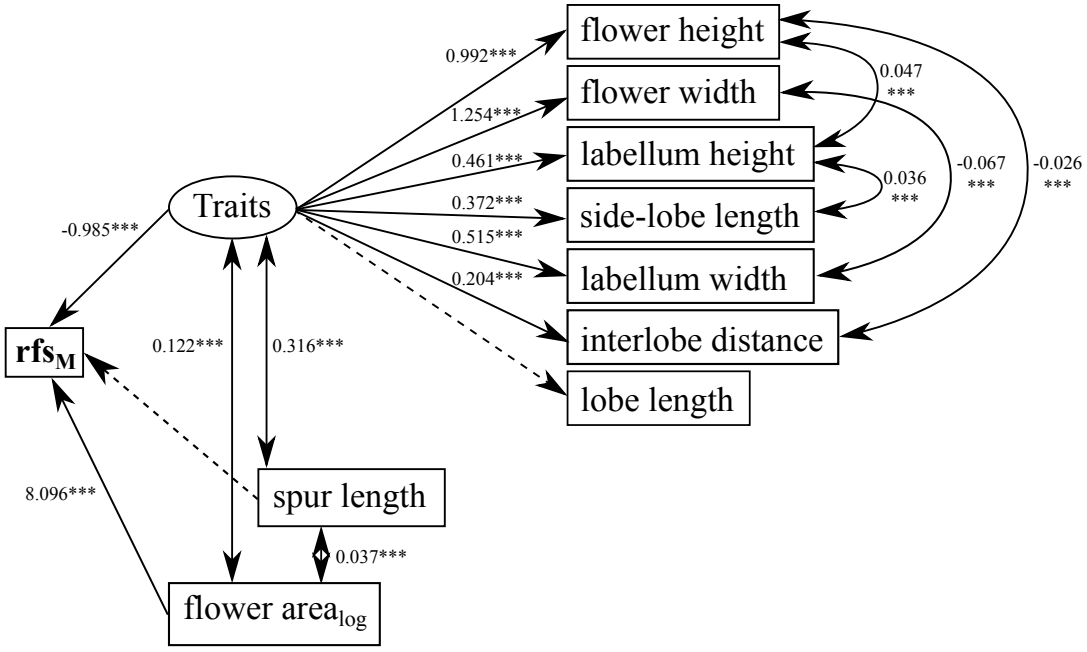


Figure 4. Structural equation model indicating the causal relationship between the indicators (shown in rectangles) and latent variable (oval) with reproductive success ('rfs'), in (A) the lowland and (B) the mountain region. In both path models, intercorrelated indicators or variables are depicted by double-headed arrows. The path coefficients from unstandardized parameters are indicated above the one-way arrows,

with only significant results of the coefficients shown, where "***": $P < 0.001$, "**": $P < 0.01$, "*": $P < 0.05$. Non-significant connections between the factors are shown with dotted lines.

Differences of floral morphology between altitudinal regions and populations

There are significant differences in morphology traits between the lowland and mountain regions (Table 3) as well as among populations (Table S4). Between the altitudinal regions, three labellum traits differ in which the lobe dimensions (lobe length and width) are significantly greater for mountain populations, while labellum width is greater in the lowland populations. Also, spur length is significantly longer in the mountain populations than in the lowlands.

Table 3. Mean ± 1 SD of the nine morphology traits of flowers in the lowland and mountain region. In bold are the trait measurements that were greater when comparing the lowland and mountain flowers. Significant differences in traits between the altitudes from the generalised linear models (GLMs) are indicated with stars, where "***": $P < 0.001$, "**": $P < 0.01$, "*": $P < 0.05$.

Traits	Trait value				$GLM_{altitude}, z_1$
	Lowland		Mountain		
	n	Mean ± SD	*n*	Mean ± SD	
(1) Flower width	446	**8.38 ± 1.17**	415	8.35 ± 1.32	2.52
(2) Flower height	446	7.25 ± 0.94	414	**7.31 ± 1.052**	0.02
(3) Labellum width	446	**3.07 ± 0.48**	412	2.99 ± 0.61	6.53 *
(4) Labellum height	447	3.47 ± 0.51	416	**3.54 ± 0.53**	0.85
(6) Side-lobe length	440	2.41 ± 0.46	408	**2.46 ± 0.46**	0.96
(7) Interlobe distance	431	1.60 ± 0.22	398	**1.63 ± 0.30**	6.53 *
(8) Spur length	446	4.28 ± 0.54	411	**4.41 ± 0.68**	13.37 ***
(9) Flower area	440	27.22 ± 6.81	403	**28.08 ± 7.25**	0.61
(10) Lobe length	441	**0.70 ± 0.57**	407	0.65 ± 0.56	14.42 ***

All units of absolute trait values are in mm, with the exception of 'flower area' (trait 9) which is measured in mm^2.

Floral Symmetry

The petal, labellum and lobe traits were found to be more symmetrical in the lowland individuals rather than in the mountains (Figure S3). However, the symmetry of all three traits are not significantly different between the regions and populations, aside from the side petals symmetry which differs between populations only (Table 4). Symmetry traits do not appear to be under significant selection in both lowland and mountains (Figure S4 and Table S5) or among populations (Table S6).

Table 4. Generalised linear model results on the effect of population and region on the side petal, labellum and major lobe symmetry ratios. Significant differences in trait ratios between the altitudes from the general linear models (GLMs) are indicated as "***": $P < 0.001$, "**": $P < 0.01$, "*": $P < 0.05$.

	Population	Region
Symmetry trait	$GLM_{population}$, z_4	GLM_{region}, z_1
petal	10.314*	0.484
labellum	7.614	3.553
lobe	4.192	0.290

Discussion

In this large-scale study with the rewarding orchid *Gymnadenia odoratissima*, we found differences in selection on floral traits between the lowland and mountain regions but not between the years. Most of the morphology traits were under stronger positive selection in the lowlands compared to the mountain region. Floral morphology differed between plants of the two regions and between populations of each region. No clear indication of selection on floral symmetry was observed. This study is one of the few examples of morphological selection over a geographical and altitudinal range, over more than one year.

Spatial structure in selection on floral phenotypes can result from variation in the preferences of (the most efficient) pollinators. However, aside from plants' antagonists such as herbivores, abiotic factors may also drive or counteract selection on floral traits (Ehrlén, 1997, Leimu et al., 2002, Gómez, 2003, Whittall and Strauss,

2006, Toräng et al., 2008, Harder and Johnson, 2009). Although these factors may introduce noise to the data or cause shifts in the direction of selection on floral morphology, it is unlikely that they influence floral trait selection nearly as much as pollinators. In particular, in our study species, it has been find in previous studies that (1) there is strong pollination limitation in most populations, a prerequisite for pollinator-mediated selection, and (2) there was low to negligible herbivory found in the study populations (Gross et al., in review). For understanding possible selection attributed to abiotic factors, a more thorough study of the correlation of environmental factors with reproductive success would be required. Thus, in light of these reasons, we discuss our study under the frame of pollinator-mediated selection.

Traits under selection

There was strong correlation between many of the morphology traits measured indicated from the factor analysis and structural equation modeling (SEM). All ten traits measured separated into two PC factors, with one factor encompasses most of the floral traits, and the other factor containing some of the labellum dimensions consisting of the major lobe (length and width) and also the labellum width. This study shows selection acting on various floral traits consistent with many previous studies. Selection has been shown to act on various floral traits measured, such as flower area or display size (e.g. Benitez-Vieyra et al., 2006, Sandring and Agren, 2009, Parachnowitsch and Kessler, 2010, Sletvold and Agren, 2010, Sletvold et al., 2010, Schiestl and Johnson, 2013), and spur length (e.g. Maad, 2000, Sletvold and Agren, 2010, Sletvold et al., 2010, Alexandersson and Johnson, 2002). The SEM on *Gymnadenia* floral traits revealed selection on both these central traits. The theoretical consensus for natural selection on larger values in floral traits is that larger flowers are more conspicuous and are associated with larger rewards, and thus selected for by pollinators (Blarer et al., 2002, Harder and Johnson, 2009, Dudash et al., 2011). In our study, there was possible selection for overall larger floral displays through selection on increased floral trait sizes, including flower area, in addition to previous studies in the same species showing positive selection on the number of flower per plant (Gross et al., in review).

Both the selection analysis and SEM in this study indicated positive selection on spur length in the lowland populations. Pollinators are responsible for selection on

spur length as an outcome of mechanical fit between the pollinator and flower in long-spurred plants. In orchids, a precise match is required between the flower and pollinator for effective pollen transfer. Previous studies in the genus *Disa* have shown a close correlation between the local pollinator tongue length and the orchid spur length (Johnson, 1994, Johnson and Steiner, 1997). Our results are consistent with many studies showing selection for longer spur or floral tube length. Selective pressures for longer spur or tube lengths can result in longer pollinator visitation time or increase contact with pollen, thus enhancing the sexual function of the flower. A study in the hawkmoth-pollinated *Gladiolus longicollis* showed positive selection on the flower-tube length, where the length directly affects pollen receipt (Alexandersson and Johnson, 2002).

With regards to floral symmetry, little significant evidence of selection has been found within populations and also regions in our study. The petal symmetry differed between the altitudes as well as the populations, however the other symmetry traits did not differ. Various previous studies have indicated that pollinators exhibit an innate preference for bilateral symmetry (e.g. Møller, 1995, Møller and Sorci, 1998, Rodríguez et al., 2004). However, evidence for bilateral symmetry is not always consistent. Some of the examples where no preference for symmetry was shown are in *Hesperis matronalis* (Brassicaceae; Weeks and Frey, 2007), *Impatiens pallida* (Balsaminaceae) by bumblebees (Frey et al., 2005), and *Gortesia diffusa* (Asteraceae) by bee flies and beetles (Midgley and Johnson, 1998).

Selection and altitudinal pollinator ecotypes

A previous study in pollinator composition of lowland and mountain pollinators has revealed distinctive differences in pollinator ecotypes between the altitudinal regions (Sun et al., 2014). As pollinators differ in functional morphology, thermal and nutrient requirements, as well as innate floral preferences, geographical variation in pollinator guilds can result in divergent selection pressures on floral traits between regions. The study found more moth pollinators in the mountain populations while butterflies are the dominant pollinators in the lowlands. Within the six traits under positive selection was the trait spur length, which was also shown in the SEM model to be positively correlated with female reproductive success in the lowland regions. In Lepidoptera, the body size or mass is commonly strong indicators of proboscis length,

with exceptions such as hawkmoths and generalist butterflies deviating from this relationship (i.e., possess greater relative proboscis length) (Corbet, 2000, Agosta and Janzen, 2005). Thus, the abundant butterflies pollinating in the lowlands are likely to have greater proboscis lengths than the small moth species dominating the mountains, inducing the positive selection detected on lowland floral spur length. However, an analysis of the correlation between proboscis length and spur length between these regions is required to confirm this.

In the mountains, selection on some of the labellum traits including the major lobe has been detected, however this was not significantly different between the regions. The SEM model indicated negative selection on most traits but positive selection on flower area. Overall, this may suggest there is little or negative selection on most traits, but selection for a wider labellum and perhaps also wider petals and sepals (width of these were not measured) which may increase the overall flower area. Due to the lack of knowledge on morphology preferences by different functional pollinator groups, correlating the traits under selection and the dominant pollinator groups may be too speculative without further investigation.

Conclusion and future prospects in phenotypic selection studies

Phenotypic selection analyses provide valuable insight into floral adaptation, but despite this there is a lack of studies in this field. A possible cause for the lack of studies in intraspecific floral morphology or selection on multiple traits and floral symmetry is in the difficulty of their quantification. Floral morphology is a complex trait in many aspects, and would in future benefit from geometric morphometric tools and analysis as oppose to linear approximations. Geometric morphometrics (GM) has been employed frequently for answering evolutionary questions based on complex phenotypes in a number of different organisms over the years (Lawing and Polly, 2010, Mitteroecker and Gunz, 2009, Schaefer and Bookstein, 2009). Unlike the distance-based morphometrics used here, GM uses the entire geometry of the flower enabling a more accurate shape analysis (Mitteroecker and Gunz, 2009), and thus should be an important tool for morphological studies in the future.

Our study has highlighted the significant influence of a geographical and altitudinal differences, and potential differences in pollinators, in morphological selection on various floral traits. Many previous studies have indicated that pollinators

are clear drivers of floral evolution on a macro-evolutionary scale, much insight could be gained from micro-evolutionary studies of trait selection between populations or geographic regions such as this current study.

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Supplementary Information

Table S1. The number of individuals used in the analyses in each population within the years 2010 and 2011.

Population	Altitude	Year	
		2010	**2011**
Döttingen	lowland	78	49
Linn	lowland	99	100
Remigen	lowland	93	30
Albula	mountain	99	-
Corviglia	mountain	-	70
Münstertal	mountain	100	50
Schatzalp	mountain	47	51

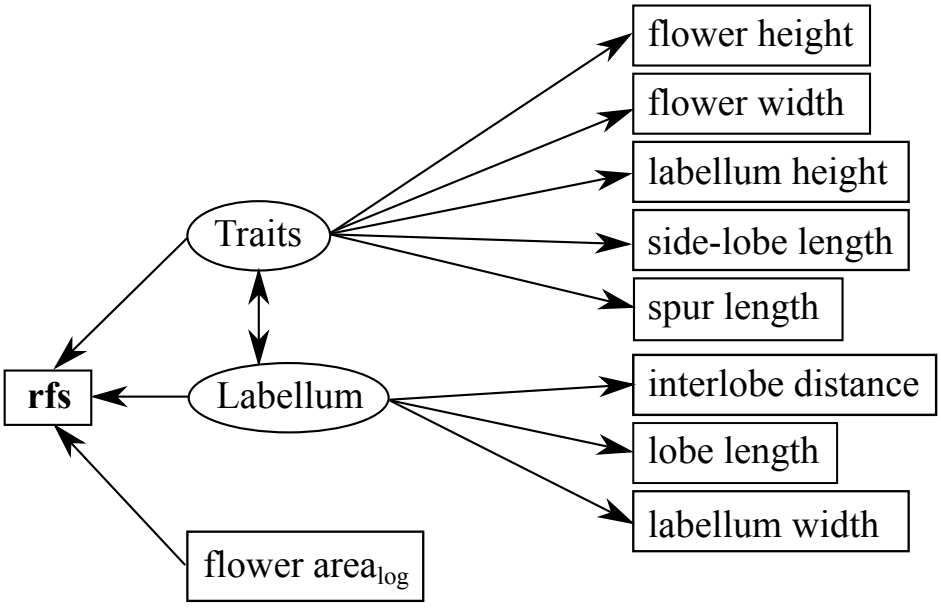

Figure S1. The initial model construct for the structural equation modelling showing the relationship between indicator variables (rectangles), latent variables (ovals) and the reproductive success of plant individuals. The latent variable groupings were constructed based on principle components. The 'labellum' variable encompasses some of major labellum variables in PC2, while the 'traits' variables includes most of the remaining morphological traits.

Table S2. The factor loadings of floral traits on principal components (PCs) with eigenvalues greater than one. The total variance explained by each of the PCs is shown in brackets beside the PC. The highest loadings are highlighted in bold.

Traits	Principle components	
	PC1 (46.51%)	PC2 (23.01%)
(1) Flower width	**0.783**	0.370
(2) Flower height	**0.877**	0.354
(3) Labellum width	0.512	**0.624**
(4) Labellum height	**0.879**	0.278
(6) Side-lobe length	**0.895**	0.067
(7) Interlobe distance	0.127	**0.860**
(8) Spur length	**0.542**	0.056
(9) Flower area	**0.802**	0.492
(10) Lobe length	0.120	**0.593**

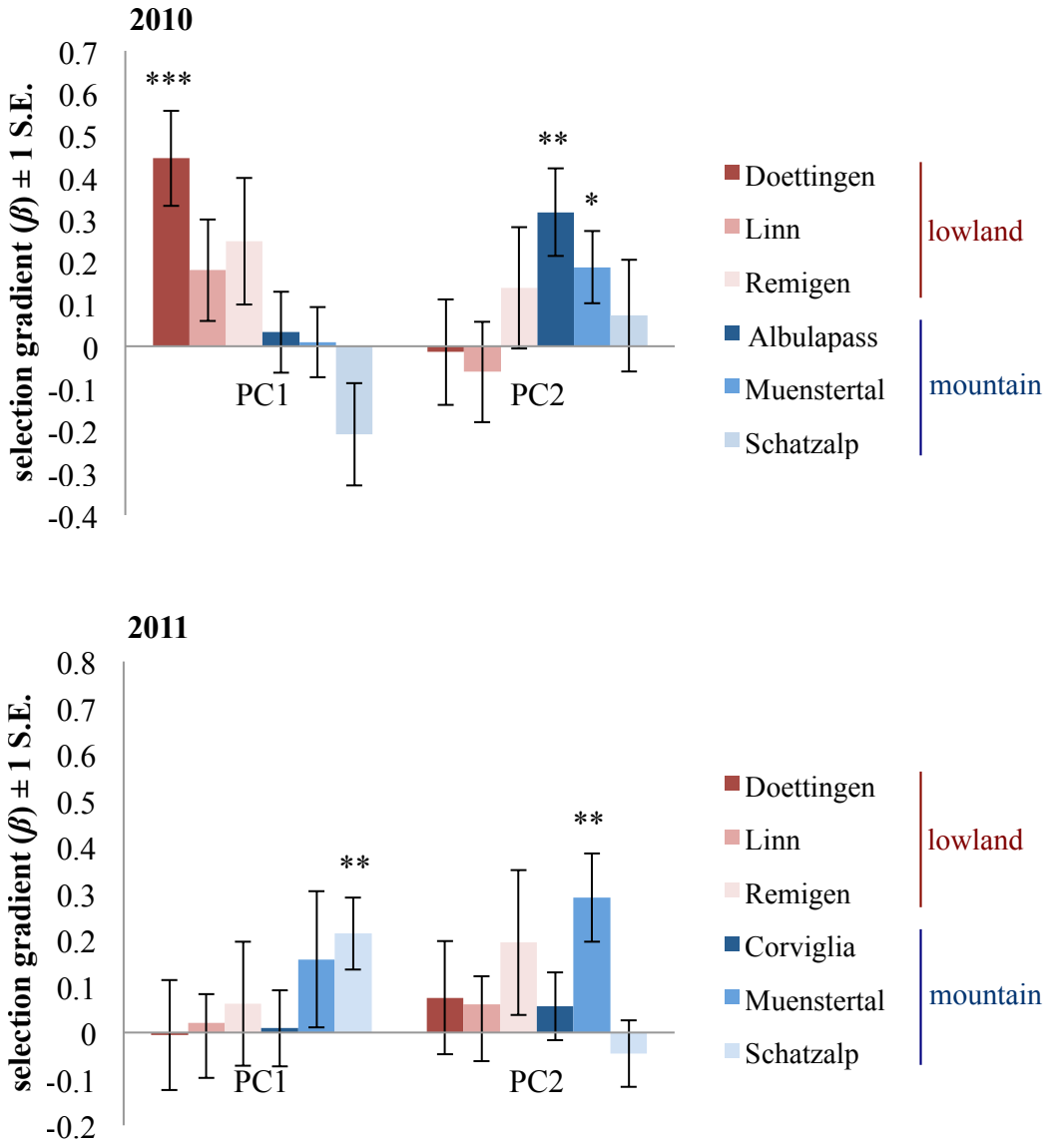

Figure S2. Linear selection gradient (β) ± 1 SE for the two principal components (PCs) of the populations of both the lowland and mountain altitudinal region in *Gymnadenia odoratissima*. Data from the three lowland populations of Döttingen (under 'Doettingen'), Linn, and Remigen ($N_{lowland}$ = 481), and the four mountain populations of Albulapass, Corviglia, Münstertal ('Muenstertal') and Schatzalp ($N_{mountain}$ = 472) were used in the analysis from the years 2010 and 2011. Significance in the linear selection gradients β are denoted above bars as "***" : $P < 0.001$, "**" : $P < 0.01$, "*" : $P < 0.05$.

Table S3. Summary of goodness-of-fit tests (χ^2), Akaike Information Criterion (AIC), Comparative Fit Index (CFI) and Root Mean Square Error of Approximation (RMSEA) of the lowland and mountain SEM models.

Model	χ^2	***df***	***P***	***AIC***	***CFI***	***RMSEA***
Lowland	286.02	27	0.00	1885.52	0.947	0.146
Mountain	375.36	28	0.00	2506.83	0.921	0.172

Table S4. Mean (± SD) of morphology trait values for all populations of the lowland and mountains. Generalised linear model comparisons between the populations are shown for each morphology trait. Traits that were significantly different are shown as "***" : $P < 0.001$, "**" : $P < 0.01$, "*" : $P < 0.05$.

Traits	GLM	Lowland populations			Mountain populations			
	Z_5	Döttingen	Linn	Remigen	Albulapass	Corviglia	Münstertal	Schatzalp
(1) Flower width	420.30 ***	9.16 ± 1.02	7.64 ± 0.76	8.77 ± 1.15	8.72 ± 0.98	7.84 ± 0.86	7.62 ± 1.31	9.46 ± 0.98
(2) Flower height	343.81 ***	7.85 ± 0.90	6.65 ± 0.62	7.57 ± 0.85	7.66 ± 0.75	6.94 ± 0.73	6.77 ± 1.13	8.03 ± 0.81
(3) Labellum width	365.07 ***	3.41 ± 0.48	2.79 ± 0.34	3.18 ± 0.42	3.09 ± 0.46	3.05 ± 0.43	2.59 ± 0.53	3.44 ± 0.58
(4) Labellum height	274.83 ***	3.79 ± 0.51	3.17 ± 0.36	3.60 ± 0.44	3.61 ± 0.38	3.34 ± 0.39	3.33 ± 0.59	3.92 ± 0.43
(6) Side-lobe length	217.44 ***	2.68 ± 0.48	2.18 ± 0.34	2.50 ± 0.40	2.59 ± 0.42	2.23 ± 0.36	2.30 ± 0.47	2.73 ± 0.39
(7) Interlobe distance	153.77 ***	1.67 ± 0.25	1.54 ± 0.18	1.63 ± 0.19	1.62 ± 0.22	1.77 ± 0.25	1.46 ± 0.26	1.77 ± 0.33
(8) Spur length	20.83 **	4.32 ± 0.67	4.26 ± 0.52	4.26 ± 0.43	4.56 ± 0.52	4.44 ± 0.58	4.24 ± 0.72	4.51 ± 0.78
(9) Flower area	435.45 ***	32.23 ± 6.69	22.71 ± 4.16	29.13 ± 5.69	29.97 ± 6.09	25.71 ± 5.28	23.78 ± 6.23	33.90 ± 6.17
(10) Lobe length	236.01 ***	0.76 ± 0.61	0.51 ± 0.52	0.94 ± 0.52	1.08 ± 0.21	0.04 ± 0.05	0.69 ± 0.51	0.61 ± 0.64

All measurements are in mm except for flower area, which is in mm^2

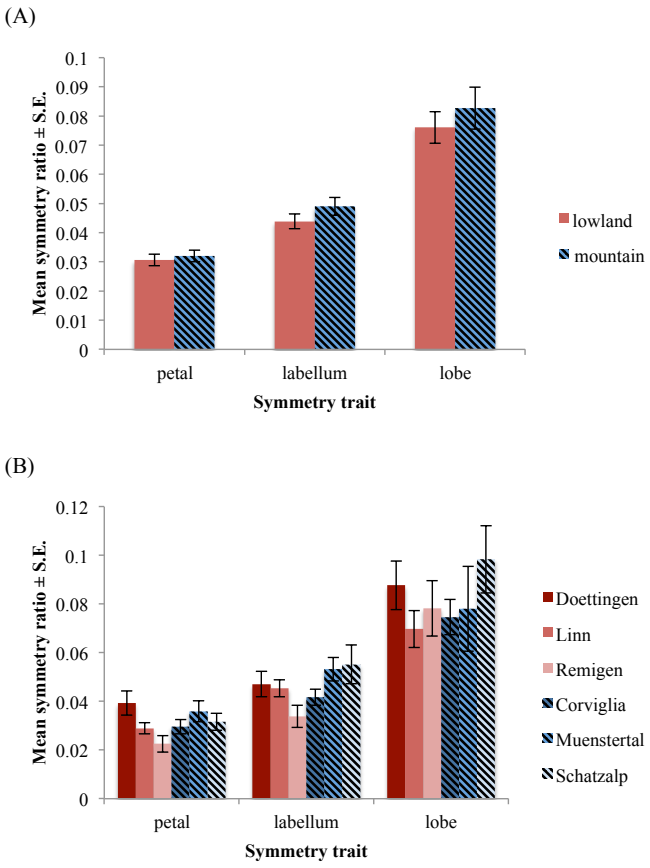

Figure S3. Mean ± S.E. symmetry ratio of the side petals, labellum and major lobe of flowers in (A) the two altitudinal regions and (B) each population. The blank, red-shaded bars are the lowland populations and the striped, blue-shaded bars are the mountain populations. A symmetry ratio of 0 suggests complete symmetry while ratios >0 suggest deviation away from complete symmetry.

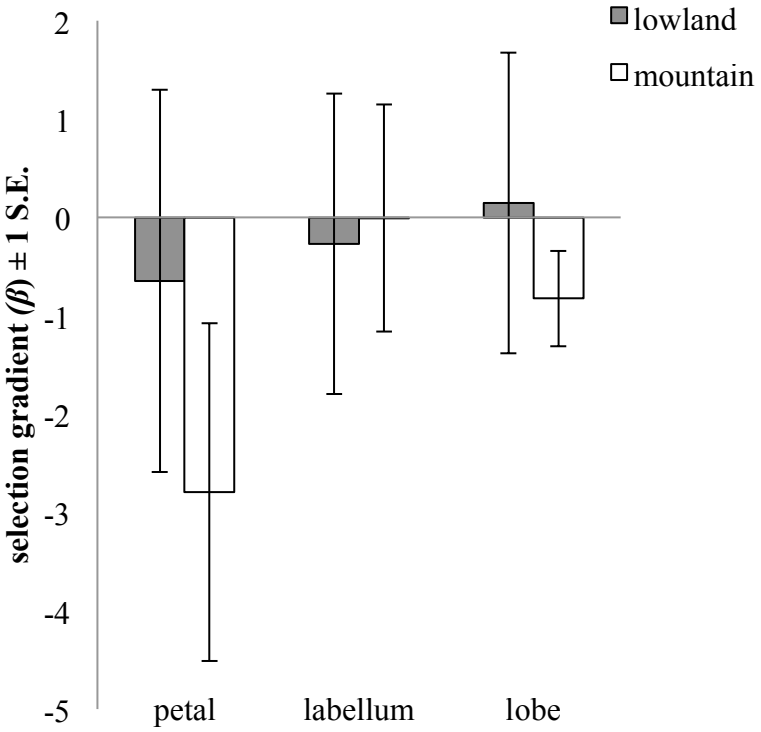

Figure S4. Differences in the linear selection gradients for the three symmetry traits of the lowland and the mountain altitudinal region in 2011. There was no significant difference in selection on the symmetry traits between the two altitudes.

Table S5. Differences in directional selection in the three symmetry trait ratios between altitudinal regions analysed through linear mixed models. Significance in the interactions are highlighted in bold.

Symmetry traits	Trait x region	
	χ^2_1	*P*
petal	0.019	0.890
labellum	0.799	0.371
lobe	0.471	0.493

Table S6. Differences in directional selection in the three symmetry trait ratios among populations for the lowland region and mountain region using linear models. Significant in the interactions are highlighted in bold.

Symmetry traits	Trait x population			
	lowland		mountain	
	F_2	p	F_2	p
petal	0.192	0.806	0.122	0.809
labellum	0.673	0.471	0.043	0.928
lobe	0.673	0.471	0.417	0.487

Chapter 2.

REGION-SPECIFIC SELECTION ON FLORAL SCENT IN A TERRESTRIAL ORCHID

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Author contributions
KG, MS and FPS designed the experiment
KG performed the majority of the field experiments, MS was also involved with the experiments
KG performed the data analysis
KG wrote the paper, contributions were made by FPS and MS

Region-specific Selection on Floral Scent in a Terrestrial Orchid

Keywords: floral signals, display size, pollinator communities, pollination, phenotypic selection, regional divergence.

Online enhancements: online appendix (including an appendix text; four appendix figures [figure A1, figure A2, figure A3, figure A4]; nine appendix tables [table A1, table A2, table A3, table A4, table A5, table A6, table A7, table A8, table A9]).

Abstract

Geographically structured phenotypic selection can lead to adaptive divergence. However, in flowering plants, a link between selection and trait divergence has not yet been shown particularly for floral signals, despite their importance for pollinator attraction. In this study, we measured phenotypic selection on display size, floral color, and floral scent in four lowland and four mountain populations of the nectar-rewarding orchid *Gymnadenia odoratissima* in two years. We also quantified spatial differences in these traits and pollinator community composition. Our results show positive selection on display size and positive, negative, or absence of selection on different scent compounds. Selection on the main scent compounds was consistently stronger in the lowlands than the mountains, and lowland plants emitted higher concentrations of most of these compounds. Pollinator community composition also differed between regions. Population differences in some scent compounds correlated with differences in selection on them. This was well complemented by more variable selection on floral scent than on display size corresponding to more variable scent emission than display size. Overall, our study is the first to document consistent regional differences in selection on floral scent, and suggests this selection pattern contributes to regional divergence in floral chemical signaling.

Introduction

One of the most intriguing characteristics of angiosperms is their striking floral diversity. Floral traits, including size, shape, color, and scent, act as visual or olfactory signals attracting pollinators (Raguso 2008; Schiestl and Johnson 2013). Adaptation to specific pollinators plays an important role in the evolution of flower diversity in angiosperms (Grant 1949; Harder and Johnson 2009; van der Niet and Johnson 2012). Pollinators show preferences towards different floral signals (Hirota et al. 2013; Newman et al. 2012; Schemske and Bradshaw Jr. 1999; Vereecken et al. 2010). Therefore, pollinators can select for floral signal divergence (Campbell et al. 1997; Gómez et al. 2008) and facilitate diversification in floral signals within and between plant species (Schiestl and Johnson 2013).

At the intraspecific level, geographically structured divergence in floral traits is common (Herrera et al. 2006). In widely distributed plant species, pollinator communities often differ regionally, particularly along altitudinal gradients (Brown et al. 2011; Brunet 2009; Bustamante et al. 2010; Malo and Baonza 2002; Price et al. 2005). Regional differences in pollinator communities, can impose divergent selection and result in complex geographically structured selection (Gómez and Perfectti 2010; Thompson 2005; Thompson and Cunningham 2002; Thompson and Fernandez 2006). In several plant species, studies suggest that divergence in floral morphology results from regional differences in pollinator communities (e.g. Hopkins et al. 2012; Johnson and Steiner 1997; Moeller 2006; Newman et al. 2012). However, in most studies, evidence for divergent natural selection as the cause for floral-trait divergence is not compelling, and trait divergence could just as well result from phenotypic plasticity or genetic drift (Herrera et al. 2006). In particular, the causes for regional divergence in floral signals, particularly floral scent, are not well understood (Majetic et al. 2008; Salzmann et al. 2007; Soler et al. 2011; Suinyuy et al. 2012).

Floral scent is a key trait for plant-insect interactions (Schiestl and Johnson 2013). Through several functional studies we know that floral scent, which is usually a complex bouquet of volatile organic compounds (VOCs), can have different functions ranging from attraction of pollinators to deterring antagonists (Huber et al. 2005; Junker and Blüthgen 2010). Floral scent often shows considerable variation both regionally on the intraspecific level and between plant species (Raguso 2008). Despite the undisputed importance of floral scent for plant reproductive success, few studies on floral trait evolution have incorporated

this trait. As a consequence, we know little about the relative importance of scent in mediating plant-pollinator interactions and its role in adaptive plant diversification.

In the present study, we measured phenotypic selection on display size, floral color, and floral scent in four lowland and four mountain populations in the orchid *Gymnadenia odoratissima*; three of these populations in the lowland and two in the mountain region were investigated in two consecutive flowering seasons. In total, 1028 plants were analyzed. This terrestrial orchid species grows over a wide altitudinal range, from lowlands to the alpine zone (Hess et al. 1976). In Switzerland, where we conducted the study, *G. odoratissima* forms locally abundant populations, making it a viable system to investigate geographically structured differences in phenotypic selection in relationship to differences in pollinator community composition. Floral signals differ considerably between lowland and mountain *G. odoratissima* plants (Sun et al. 2014). *Gymnadenia odoratissima*, which produces nectar in a short floral spur, has a functionally specialized pollination system, i.e. it is specialized to a functional group of pollinators, and attracts a range of primarily lepidopteran pollinators (Huber et al. 2005; Sun et al. 2014; van der Cingel 1995). We addressed the following questions to elucidate whether temporally consistent geographically structured, divergent selection influenced the observed differentiation in floral signals: (i) Which floral signals are under phenotypic selection in the lowlands and which in the mountains? (ii) Does selection on floral signals differ geographically, particularly between the lowland and mountain region, and, if so, how consistent is this difference between years? (iii) Does spatial and/or temporal variation in selection differ between different floral signals? (iv) Do differences in selection correlate with differences in floral signals?

Materials and Methods

Study Species and Populations

The terrestrial orchid *Gymnadenia odoratissima* (L.) L.C.M. Rich. (common name: Short-Spurred Fragrant Orchid) has a geographic distribution restricted to the temperate zone of Europe (Gustafsson and Sjögren-Gulve 2002; Hultén and Fries 1986) and grows almost exclusively on calcareous soil from the lowlands to the alpine zone (Hess et al. 1976). In Switzerland, where we conducted the study, *G. odoratissima* occurs within an altitudinal

range from 300 to 2400 m a.s.l. and grows in locally abundant populations. Each plant forms a single inflorescence consisting of approximately 10-140 flowers. Floral color ranges from dark purple to pale pink in the lowlands and from pink to white in the mountains. The species is self-compatible but largely outcrossing and the strong, sweet floral scent is important to attract pollinators (Huber et al. 2005). Seven scent compounds (mostly aromatics) were shown to elicit EAD responses in pollinator insects, and one of these compounds (phenylacetaldehyde) was also found to attract pollinators in the field (Huber et al. 2005). We conducted the present study in eight natural populations – four lowland populations in north-eastern Switzerland (Döttingen, Remigen, Linn, and Rossweid; 500-650 m a.s.l.) and four mountain populations in south-eastern Switzerland (Schatzalp, Münstertal, Albulapass, and Corviglia; 1800-2250 m a.s.l.) – and between 2010 and 2012 (table A1).

Measurement of Floral Signals

When most plants were in full flower in a population (lowland: end of June to mid-July, mountain: mid-July to mid-August), we marked individual plants within approximately 2 m of transects. Plants were marked in three lowland and three mountain populations in 2010 and in four lowland and three mountain populations in 2011; in three of these populations in the lowlands and in two in the mountains, we marked plants in both years (table A1). In each population and year, 100 plants were marked, except in the lowland population Remigen (60 plants) and the mountain population Schatzalp (99 plants) in 2011 (table A1). Two to four days were needed to measure the floral signals of all marked plants in a population.

We measured plant height (ground to uppermost flower) and inflorescence length (calculated as the difference between plant height and stem length [ground to lowermost flower]) to the nearest centimeter using a measuring tape. In addition, we counted the total number of flowers. Plant height, inflorescence length, and the total number of flowers were used as a measure of display size in further analyses.

We collected floral scent for 30 min at some time between 9:00 a.m. and 7:00 p.m. on days without rain using headspace sorption, a completely non-invasive method that does not damage the plant from which scent is collected. Due to the large number of plants ($n > 1000$ plants), and the length of time required for scent collection, it was not possible to collect scent at the same time for all plants; diurnal changes in scent emission are, however, not strong in *G. odoratissima* (unpublished data). We enclosed the inflorescence of each individual in an oven bag (Nalophan®) tied closed with short pieces of florist wire. A small glass tube, filled

with approximately 20 mg 80/100 mesh Tenax® absorbent powder (Supelco, Bellefonte, PA, USA; called "filter" hereafter), was inserted into each bag. The filter was connected to a battery-operated vacuum pump (PAS-500 Micro Air Sampler, Spectrex, Redwood City, CA, USA) using a silicone tube. Air was vacuum pumped out of the bag through the filter at a rate of 150 ml min^{-1}, trapping the floral volatiles on the Tenax® adsorbent. We collected air from one to two empty bags per population to control for contaminants from the surrounding air. After scent collection, we wrapped the filters' ends with PTFE (Teflon®) thread seal tape and packed each individual filter in aluminum foil or in a small glass vial. Filters were stored in a -30 °C freezer until analysis. Samples were analyzed by gas chromatography with mass selective detection (GC-MSD). A thermal desorption system (TDS; TDS3, Gerstel, Mühlheim an der Ruhr, Germany) with a cold injection system (CIS; CIS4, Gerstel, Mühlheim an der Ruhr, Germany) was used to inject samples into an Agilent GC 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), which was equipped with an HP-5 column (0.25 mm diameter, 0.32 µm film thickness, 30 m length), and helium was used as carrier gas at a flow rate of 1.9 ml min^{-1}. The TDS temperature was programmed to rise from 30 °C (0.5 min hold) to 240 °C (1 min hold) at 60 °C min^{-1} for thermal desorption; the CIS temperature was -150 °C during thermal desorption and was programmed to rise from -150°C (0.5 min hold) to 150 °C at 16 °C s^{-1} and from 150 °C to 250 °C (0.5 min hold) at 12 °C s^{-1} for injection. The GC oven temperature was programmed to rise from 50 °C to 230 °C at 8 °C min^{-1}. The GC was connected to an Agilent MSD 5975 mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Compounds were identified by comparing obtained mass spectra with those from the NIST spectral reference database (NIST 05) implemented in the ChemStation Enhanced Data Analysis program (G1701EA E.02.02 MSD Productivity ChemStation Software, Agilent Technologies, Germany). Compound identification was verified by comparing obtained mass spectra with those of synthetic standard compounds. One or two concentrations of these synthetic standard compounds were analyzed to obtain calibration curves using the peak area of a compound-specific qualifier ion. The calibration curves were employed to convert the peak areas of the compound-specific qualifier ions in the *G. odoratissima* samples into nanograms using the ChemStation program. We manually double-checked all samples and compounds and, if necessary, manually integrated the peak area. For analysis, we calculated compound amounts in nanograms per liter sampled air per inflorescence. We included a compound as floral scent compound when it met the following criteria: (i) its median concentration for air controls was lower than 80% of its mean concentration for plant samples in the corresponding population for at least one year, and (ii)

its mean concentration for plant samples was higher than 0.5 ng l^{-1} per inflorescence in both years. Applying these criteria resulted in a list of 22 floral scent compounds.

Floral color was only measured in 2011. Due to time constraints during field work, floral color was quantified as categorical intensity rather than by spectrophotometry. We cut off two open flowers per individual (one from the bottom and another from the top of the inflorescence), placed them on a white paper, and photographed them. We determined five flower photographs as standards according to 1 "white", 2 "pinkish white", 3 "light pink", 4 "pink", and 5 "purple". The color of all other photographed flowers was classified on a computer screen using these standards. This simplified color assessment is justifiable in *G. odoratissima* as spectral reflectance of its flowers is restricted to the wavelength range visible for the human eye, and floral color differences primarily result from differences in the relative spectral reflectance in the wavelength range between 488 nm and 636 nm (Sun et al. 2014).

Female Reproductive Success

When fruits were mature, we counted the number of fruits generated by the plants used to measure floral signals. Some plants or labels were missing due to browsing herbivores and mowing. Fitness was estimated by calculating relative female reproductive success (fRS) as the number of fruits produced by an individual divided by the mean number of fruits produced by the marked plants in the same population and year. In addition, the proportional fRS was calculated as the number of fruits divided by the total number of flowers for each individual plant. Female reproductive success generally differed between populations both in the lowland and the mountain region, but it generally did not differ between altitudinal regions (appendix text; table A2). In our study, it was not feasible to assess male reproductive success, as this would have required multiple scoring of pollinia removal rates during the flowering season.

Pollen Limitation

Pollen limitation and dependency on pollinators was examined by conducting a pollination experiment with three treatments: hand pollination, pollinator exclusion, and open pollination as a control. For the hand-pollination treatment, we supplementally hand-pollinated the flowers of 4-10 plants per population in the lowland population Döttingen and the mountain populations Schatzalp and Albulapass in 2010 and in the lowland populations Döttingen,

Remigen, Linn, and Rossweid and the mountain populations Schatzalp, Münstertal, and Corviglia in 2011 (table A1). We pollinated flowers with pollinaria collected from conspecific plants at least five meters away, using wooden toothpicks or tweezers. The hand-pollinated plants were marked and bagged with fine-meshed wire insect nets (tesa® AG, Hamburg, Germany) to prevent further pollinator visitations. For the pollinator-exclusion treatment, we bagged four plants in the lowland population Döttingen and the mountain population Albulapass in 2010 to prevent pollinator visitations. Plants used for floral signal quantification (see above) served as the untreated open-pollination control plants with free access to natural pollinator visitations. When fruits were mature, we counted the total number of flowers and fruits produced per inflorescence to calculate the proportional fRS. For each population and year, we calculated the extent of pollen limitation as 1 – (mean proportional fRS of open-pollinated control plants/mean proportional fRS of hand-pollinated plants) (Sletvold and Ågren 2010).

Pollinator Community Composition and Floral Herbivory

We assessed pollinator community composition in all populations where we quantified floral signals. *G. odoratissima* pollinators were observed and caught between 7:00 a.m. and 11:00 p.m. and between 29 June and 09 August in 2010, 2011, and 2012 by slowly walking through the populations. Insects were considered pollinators if they probed *G. odoratissima* flowers and either removed pollinaria or, in case pollinaria were already removed, exhibited behavior likely leading to pollinaria removal. Additionally, insects that rested on *G. odoratissima* inflorescences and carried pollinaria of the size of *G. odoratissima* pollinaria were considered pollinators. For later identification, pollinators were caught using hand nets, transferred to individual plastic tubes with air holes, and stored at -30 °C until preparation; alternatively, we photographed the pollinators in the field. When at least one individual of a pollinator species had previously been caught or photographed, we only recorded the observation. For each observed pollinator, we documented the population, date, and observation time and noted whether the pollinator carried pollinaria on its proboscis. Specimens could not always be identified to the species or genus level; therefore we conducted statistical analyses at the family level for Lepidoptera and order level for other insects. We only considered populations where we caught and/or observed pollinators on at least two different days; these were the lowland populations Döttingen (5 d), Remigen (5 d), Linn (3 d), and Rossweid (2 d) and the mountain populations Schatzalp (12 d), Albulapass (2 d), and Corviglia (2 d). Median total

observation time per population did not differ between the lowland (18.00 h) and mountain (19.25 h) region (Kruskal-Wallis test: d.f. = 1, test statistics = 0.000, $P = 1$), but differed considerably between populations (lowland region: 27.00 h in Döttingen, 19.75 h in Remigen, 14.50 h in Linn, 16.25 h in Rossweid; mountain region: 37.50 h in Schatzalp, 2.00 h in Albulapass, 19.25 h in Corviglia). These differences were accounted for by calculating the total number of pollinators and number of pollinator families/orders caught and observed per hour. The number of pollinators per hour served as a measure of visitation rate and the number of different pollinator families/orders per hour as a measure of pollinator richness. The similarity of pollinator communities between populations and altitudinal regions was quantified by computing the Bray-Curtis dissimilarity as $(\Sigma (|y_{i1} - y_{i2}|)) / (\Sigma (y_{i1} + y_{i2})$ (Bray and Curtis 1957) for each pair of dates per population using the package *ecodist* (version 1.2.7 (Goslee and Urban 2007)) in the statistical software program R (version 3.0.1 (RCoreTeam 2013)).

As we observed some floral herbivory in the form of eaten flowers and aphid infestation, we quantified the magnitude of floral herbivory for all marked plants on the day we measured floral signals as described in the appendix text. Floral herbivory was generally low in our study populations (figure A1). It generally differed between populations in both altitudinal regions, but did not differ between the lowland and the mountain region (figure A1).

Statistical Analyses

For most statistical analyses, we used SPSS 20.0.0.0 for Windows (IBM SPSS Statistics, IBM Corp., Released 2011, Armonk, NY, USA). R (RCoreTeam 2013) was used for the remaining statistical analyses (as stated in the text) due to its suitability for specific analyses.

Phenotypic Selection Analyses

Display size and floral scent were measured in 2010 and 2011, and floral color was measured only in 2011. Therefore, phenotypic selection analyses were conducted in two ways (i) in a two-year data set (2010 and 2011), containing display size and floral scent, and (ii) in a one-year data set (2011), which included display size, floral scent, and floral color. Results from the two analyses did not differ for display size and floral scent; therefore we describe the statistical analyses and report results for the two-year data set and provide the results for the analyses on the 2011 data set in the appendix (Figs. A2, A3).

A principal component analysis (PCA) was performed on all floral display and floral scent traits as well as all populations and both study years to reduce the number of variables and convert the potentially correlated variables into linearly uncorrelated principal components (PCs). The PCA was conducted with traits standardized to a mean of 0 and a standard deviation (SD) of 1 for each population. For floral scent, we used amounts per inflorescence per liter sampled air. We extracted principal components (PCs) with an eigenvalue > 1 using varimax rotation (an orthogonal rotation minimizing the number of variables with high loadings on each PC, which simplifies the interpretation of the PCs). This procedure resulted in seven PCs: one "display size PC" (PC3) and six "floral scent PCs" (PC1, PC2, PC4, PC5, PC6, PC7) explaining 71.8% of the total variance (table A3). Trait-value distributions of the PCs are shown in figure A4. These PCs were used as explanatory variables in the phenotypic selection analyses. Preliminary analyses did not indicate quadratic selection; therefore we assessed only linear selection in the final analysis. To determine which floral signals were under selection, we estimated selection gradients β on each PC, using linear multiple regression analyses in R (RCoreTeam 2013; Lande and Arnold 1983). Relative fRS was used as response variable and PCs as explanatory variables. To assess whether selection on PCs differed between altitudinal regions and/or years, we used a linear mixed model using the package *lme4* (Bates et al. 2013) in R (RCoreTeam 2013). The model included relative fRS as the response variable, PCs as covariates, altitudinal region, year, interactions between each PC and altitudinal region, and interactions between each PC and year as fixed factors, and population nested within altitudinal region as random factor. In addition, we conducted linear models in R (RCoreTeam 2013) to test for differences in selection among populations within altitudinal regions and between years. In these models, we included relative fRS as the response variable, PCs as covariates, and population, year, interactions between each PC and population, and interactions between each PC and year as fixed factors.

To compare the magnitude of spatial and temporal variation in selection between the two signal groups – display size and floral scent – that were quantified in both study years, coefficients of variation (CVs; standard deviation divided by the mean) of the selection gradients on PCs were used. For each PC, we calculated a value of spatial and a value of temporal variation in selection. To obtain the value of spatial variation in selection, CVs were calculated for each year and altitudinal region using population-specific selection gradients as replicates, and absolute values of these CVs were averaged across years and across regions. Similarly, to obtain the value of temporal variation in selection, CVs were calculated for each

population using year-specific selection gradients as replicates, and absolute values of these CVs were averaged across populations within altitudinal regions and then across regions. The CVs of selection on floral scent PCs were compared to the CVs of selection on the floral display PC using one-sample Wilcoxon signed rank tests. These CVs analyses were also conducted separately for the two altitudinal regions. The results were the same for the lowland and the mountain region as well as when both altitudinal regions were combined. Therefore, we only report the results for both altitudinal regions combined.

Differences in Floral Signals and Association Between Selection and Floral Signals

To assess geographic differences in floral signals, we conducted general linear models with altitudinal region, year, and population nested within region as explanatory variables. For this analyses, all trait values were ln(x + 1) transformed. In addition, to compare within- and among-population trait variation between the three floral signal groups display size, floral scent, and floral color, CVs were calculated for each trait within and between populations by dividing within- and among-population SD by each respective mean. Means of CVs were calculated between years and altitudinal regions. We analyzed the differences in the CVs between floral signal groups using Mann-Whitney U tests (display size versus floral scent) or one-sample Wilcoxon signed rank tests (display size and floral size versus floral color).

To assess correlations between differences in floral signals and differences in selection gradients on these signals, Mantel tests with 1000 permutations were conducted using the package *vegan* (version 2.0-9 (Oksanen et al. 2013)) in R (RCoreTeam 2013). Floral-signal differences were calculated as Euclidean distances between population means (means of the 2010 and 2011floral signal values) for display size (plant height, inflorescence length, number of flowers), floral color (color intensity code), overall floral scent (all 22 floral scent compounds), and each group of floral scent compounds that exhibited the highest loadings on individual PCs (table A3). Data standardized (mean ± SD of 0 ± 1) for each year were used for the Euclidean distance calculations. Similarly, selection gradient differences were calculated as Euclidean distances between population means of selection gradients (means of the 2010 and the 2011 selection gradients) for each PC. We included only populations we measured in both years; these were the lowland populations Döttingen, Remigen, and Linn and the mountain populations Schatzalp and Münstertal. All Euclidean distances were calculated using the package *ecodist* (version 1.2.7 (Goslee and Urban 2007)) in R (RCoreTeam 2013).

Pollen Limitation

Pollen limitation was tested by comparing the proportional fRS between the hand-pollination and the open-pollination treatment using a Mann-Whitney U test for each year and population. Similarly, we compared the proportional fRS between the pollinator-exclusion and the open-pollination treatment to test for pollinator dependency.

Pollinator Community Composition

Differences in pollinator community composition between the lowland and mountain region and between populations within altitudinal regions were tested by conducting PERMANOVAs (Permutational Multivariate Analysis of Variance, an ANOVA using Bray-Curtis dissimilarity data and permutation tests with pseudo-*F* ratios) using the package *vegan* (version 2.0-9 (Oksanen et al. 2013)) in R (RCoreTeam 2013). We generated a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarities employing the PROXSCAL procedure with ordinal proximity transformation and Torgerson as initial configuration to visualize differences. We conducted SIMPER (Similarity Percentages) analyses based on Bray-Curtis dissimilarities to determine which pollinators characterized the differences between altitudinal regions and between populations within altitudinal regions using the package *vegan* (version 2.0-9 (Oksanen et al. 2013)) in R (RCoreTeam 2013). Differences in pollinator visitation rates and pollinator richness between the two altitudinal regions and between populations within altitudinal regions were analyzed with Kruskal-Wallis tests.

Results

Phenotypic Selection

Phenotypic Selection at the Regional (Altitudinal) Level

For both altitudinal regions and years, display size (principal component [PC] 3), and several floral scent PCs were under significant directional selection (figure 1). Display size (PC3) was under positive selection in both regions and years. Three of six floral scent PCs (PC1, PC2, PC4) were under positive selection in at least one region and year. Floral scent PC5 was under negative selection in the lowland in 2010, and floral scent PC4 was under negative selection

in the mountain region in 2011. Floral color and two aromatic compounds (PC7A, named "floral color PC" hereafter; assessed in a separate analysis that only contained the 2011 data set; table A4), was under negative selection in both altitudinal regions (figure A2).

We found consistent differences in directional selection between the lowland and the mountain region (figure 1; table 1). Selection on floral scent PC1 and PC2 was stronger in the lowland compared to the mountain region (figure 1; table 1). In contrast, selection on display size (PC3) did not differ between the two regions, but was stronger in 2011 than 2010. Selection on floral scent PC5 also differed between years, but not between regions. Selection on the floral color PC (PC7A) did not differ between regions (figure A2).

Phenotypic Selection at the Population Level

Similar to the regional level, we found significant directional selection at the population level (figure 2). Display size (PC3) was under positive selection in all populations in both study years. In contrast, for floral scent, only floral scent PC1 was under positive selection in some populations for both years in the lowlands, but three of six floral scent PCs (PC1, PC4, and PC7) were under positive or negative selection in at least one population and year in the mountains. Floral color (PC7A) was under negative selection in only one mountain population (figure A3).

Selection on none of the PCs differed consistently between population without differing between the two study years (figure 2; table 2). Selection on display size (PC3) differed between mountain populations, but was stronger in 2010 than in 2011 in the lowland populations; and floral scent PC7 differed between populations, but also between the two years in the mountain region (figure 2; table 2). Selection on floral color (PC7A) did not differ between populations (figure A3).

Selection on floral scent was consistently more variable than selection on display size. For among-population variation in selection, coefficients of variation (CVs) for selection gradients on floral scent PCs ranged from 0.78 to 13.58 (median = 3.53), which was significantly higher than the CVs for selection gradients on the display size PC (0.21; $n = 6$, standardized test statistics = 2.201, $P = 0.028$). Similarly, for temporal variation in selection, the CVs for selection gradients on floral scent PCs ranged from 0.42 to 8.89 (median = 3.12), which was significantly higher than the CVs for selection gradients on the display size PC (0.22; $n = 6$, standardized test statistics = 2.201, $P = 0.028$).

Differences in Floral Signals and Association Between Selection and Floral Signals

Display size, floral color, and most floral scent compounds significantly differed between altitudinal regions as well as between population and between years (table A5). Among the seven compounds constituting PC1, four were emitted in higher amounts in lowland plants. Those four compounds constituted approximately 30% of the total amounts of floral volatiles emitted by the plants (table A5). The total amount of scent did not differ between lowland and mountain plants (table A5).

Floral scent was significantly more variable both within and between populations compared with floral color and display size (table A6). We found a high correlation between Euclidean distances in floral signals and selection gradients for some floral scent PCs (figure 3), even though it was significant only for floral scent PC4 and floral scent PC5 (figure 3*D* and *E*).

Pollen Limitation

Pollen limitation ranged from 0.38 to 0.63 (median = 0.51) in 2010 and from 0.10 to 0.76 (median = 0.42) in 2011. The proportional fRS was significantly higher in hand-pollinated compared with open-pollinated plants in two of three populations in 2010 and in six of seven populations in 2011 (table A7). This indicates that *G. odoratissima* is strongly pollen limited in most natural populations. Moreover, in plants from which pollinators were excluded the proportional fRS was very low (Döttingen: 0.00-1.59%; Albulapass: 0.00-3.85%), and significantly lower than in open-pollinated plants (Döttingen: $n_{open} = 75$, $n_{bagged} = 4$, $z = 2.886$, $P = 0.004$; Albulapass $n_{open} = 85$, $n_{bagged} = 4$, $z = 2.615$, $P = 0.009$), suggesting that spontaneous self-pollination is very low and plants largely depend on pollinators to set fruits.

Differences in Pollinator Community Composition

We identified a total of 196 pollinators from three insect orders: primarily Lepidoptera, which were represented by 13 families, some Diptera (all intact specimens were identified as Empididae), and few Coleoptera (table A8). Pollinator community composition differed significantly between altitudinal regions (pseudo-$F_{1,5} = 3.289$, $P = 0.030$). Lycaenidae, Zygaenidae, Crambidae, Nymphalidae, and Diptera contributed most to the differences between lowland and mountain pollinator communities and were more common in the

mountain region, with Zygaenidae and Diptera being exclusively found in the mountain populations (figure 4*A*; table A9). At the population level, pollinator community composition differed significantly between mountain populations (pseudo-$F_{2,14}$ = 2.147, P = 0.008) and, though still significant, to a lesser extent between lowland populations (pseudo-$F_{3,11}$ = 1.851, P = 0.028; figure 4*A*). Noctuidae, Hesperiidae, Nymphalidae, Pyralidae, Pterophoridae, Pyralidae, and Coleoptera were the primary contributors to differences between lowland pollinator communities, and Zygaenidae, Lycaenidae, Crambidae, and Nymphalidae primarily contributed to differences between mountain pollinator communities (table A9). Moreover, pollinator visitation rate and pollinator richness were significantly higher in mountain populations compared to lowland populations and significantly differed between mountain but not between lowland populations (figure 4*B* and *C*).

Discussion

In flowering plants, regional divergence in floral traits is widespread. A possible explanation is consistent region-specific selection. Our large-scale phenotypic selection study in the rewarding orchid *Gymnadenia odoratissima* detected strong selection both on display size and floral scent, but only weak or no selection on floral color. Our results show that only selection on floral scent differed consistently between regions. Moreover, locally variable selection explained some of the population divergence in floral scent, but not in display size and floral color. Together, our study shows variable selection dynamics, particularly on olfactory floral signals, and is the first documentation of region-specific selection on floral scent.

In animal-pollinated angiosperms, visual and olfactory signals play an important role in pollinator attraction (Raguso 2008; Schiestl and Johnson 2013). Pollinator-mediated selection is expected on both signal types, however phenotypic selection on visual signals is better documented. For example, selection for larger displays has commonly been found (Maad 2000; Reynolds et al. 2010; Schiestl et al. 2011; Sletvold and Ågren 2010; Sletvold et al. 2010). Our findings are consistent with these studies, suggesting that pollinators often prefer large displays, either because larger displays contain more reward or are better visible (Dudash et al. 2011). While some studies report selection on floral color (Caruso et al. 2010; Renoult et al. 2013), others found no evidence for it (Parachnowitsch et al. 2012). In our study, weak selection for lighter-colored flowers in *G. odoratissima* might be imposed by

nocturnal pollinators, which are known to visit *G. odoratissima* (Huber et al. 2005; Sun et al. 2014) and are better able to detect these flowers (Kelber et al. 2003). Selection studies that included floral scent are scarce; nevertheless, three recent studies reported significant but compound-specific selection on floral scent (Ehrlén et al. 2012; Parachnowitsch et al. 2012; Schiestl et al. 2011). This finding is congruent with our results and suggests floral scent is often under selection mediated by biotic interactions.

Regional divergence in floral traits is common and has been documented in morphology and floral signals (Dötterl et al. 2005; Hopkins et al. 2012; Johnson and Steiner 1997; Majetic et al. 2008; Mant et al. 2005; Newman et al. 2012; Salzmann et al. 2007). In principle, such divergence can result from phenotypic plasticity, genetic drift, or natural selection (Herrera et al. 2006). Indeed, consistent spatial variation in selection is expected to result in adaptive population divergence, if the variation in traits under selection has a heritable component (Hall and Willis 2006; Siepielski et al. 2013). In our study, we found consistent region-specific differences in selection on the major floral scent compounds. Floral scent has recently been shown to have considerable heritability (study in *Brassica rapa*; Zu and Schiestl unpublished). Thus, consistent selection differences may lead to adaptive divergence among regions. Some of our analyses indeed indicated that selection plays a role in shaping floral signal differences. As direct evidence, for some floral scent compounds, among-population Euclidean distances in selection gradients showed a correlation with among-population Euclidean distances in the same floral scent compounds. This shows that the degree of population differences in selection corresponded to differences in the respective scent compound groups. Such a correlation was not detected for display size. As more indirect evidence, high scent variability corresponded with higher among-population variability in selection on floral scent, compared to display size. Moreover, the stronger directional selection on PC1 in the lowlands, representing the major floral scent compounds, was matched by a stronger emission of five of these compounds in the lowlands. Interestingly four of these five compounds (phenylacetaldehyde, benzyl acetate, phenylethyl acetate, and eugenol) were shown earlier to elicit EAD responses in pollinators of *G. odoratissima* (Huber et al. 2005). Phenylacetaldehyde has also been shown to attract pollinators of *G. odoratissima* (Huber et al. 2005), as well as of several other plant species (Andersson et al. 2002), supporting a key function of this VOC in pollinator attraction. The consequence of spatially-varying selection, namely local adaptation, has recently been documented in *Gymnadenia odoratissima*, where mountain plants were found to be adapted to local pollinators (Sun et al.

2014). In that study, mountain plants suffered significantly reduced reproductive success when transferred into lowland populations.

Geographically structured selection on floral signals can result from consistent spatial differences in preferences of (the most efficient) pollinators towards floral signals (Hirota et al. 2013; Newman et al. 2012; Schemske and Bradshaw Jr. 1999; Vereecken et al. 2010). Additionally, antagonists, such as florivores, or abiotic selection agents, which are also likely to vary geographically, can counteract or reinforce pollinator-mediated selection (Ehrlén et al. 2012; Harder and Johnson 2009; Sandring and Ågren 2009; Strauss and Whittall 2006). Our results suggest variable, pollinator-mediated selection in *G. odoratissima.* First, fruit set was strongly pollen limited in most populations, a prerequisite for pollinator-mediated selection. Second, the pollinator community composition differed between altitudinal regions and between populations. Third, the extent of herbivory was also region- and population-specific, but it was generally low. Our study was, however, not specifically designed to test for the agent of locally variable selection and further experimental approaches are necessary to conclusively identify the agent(s) of selection.

In conclusion, our study suggests that spatial variation in average selection was consistent enough to create geographically structured selection, leading to regional divergence in floral traits. Our data emphasize the importance of measuring phenotypic selection in multiple populations and in different flowering seasons to capture the actual selection dynamics acting within a species. Furthermore, our results provide indication that geographically structured differences in selection do not act equally on all traits, suggesting different evolutionary forces acting on different traits and possibly different evolutionary rates in different traits. While our study implies ongoing microevolutionary processes in *G. odoratissima* floral signals, which influence pollinator attraction, future studies should quantify phenotypic selection on floral-morphology traits affecting pollinator efficiency, such as floral spur length, to reach a more conclusive understanding of the evolution of flowers as a whole.

Online appendix

Online Appendix Text

Measurement of Floral Herbivory

We quantified the magnitude of floral herbivory for all marked plants on the day we measured floral signals. In both years, we counted the number of flowers eaten by florivores. We observed that *G. odoratissima* inflorescences were infested by a variable number of aphids. Therefore, in 2011, we additionally quantified the aphid load on the inflorescences in six categories with 1 being no aphids and 6 many aphids. Moreover, we computed the proportion of plants with at least one flower eaten and the proportion of plants with aphids on the inflorescence for each population and year.

Statistical Analysis: Reproductive Success and Floral Herbivory

Differences in reproductive success and floral herbivory between lowland and mountain plants were analyzed using linear mixed models with altitudinal region as fixed factor and population nested within the altitudinal region as random factor for each year using the package *lme4* (Bates et al. 2013) in R (RCoreTeam 2013). Differences in reproductive success and herbivory among populations were analyzed by linear models with population as fixed factor for each year and altitudinal region in R (RCoreTeam 2013). We square root transformed the number of fruits and arcsine (2011) or arcsine square root (2010) transformed the proportional fRS to improve normality and homogeneity of variances.

Literature Cited

Bates, D., M. Maechler, and B. Bolker. 2013. lme4: Linear mixed-effects models using S4 classes. R package version 0.999999-2.

RCoreTeam. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Online Appendix Tables

Table A1: Geographic locations of the four lowland and the four mountain study-populations of *Gymnadenia odoratissima* and year(s), in which selection and floral signals was measured and hand pollinations were conducted.

Population (code)	Geographic coordinates	Altitude (m a.s.l.)	Selection and floral signals (*n*)	Hand pollination (*n*)
Lowland region				
Döttingen (D)	47°34’30’’N, 08°16’25’’E	500	2010 (100), 2011 (100)	2010 (4), 2011 (9)
Remigen (R)	47°31’45’’N, 08°09’45’’E	600	2010 (100), 2011 (60)	2011 (8)
Linn (L)	47°28’35’’N, 08°07’00’’E	500	2010 (100), 2011 (100)	2011 (9)
Rossweid (RW)	47°18’45’’N, 08°30’40’’E	650	2011 (100)	2011 (10)
Mountain region				
Schatzalp (S)	46°48’20’’N, 09°49’30’’E	1800	2010 (100), 2011 (99)	2010 (5), 2011 (10)
Münstertal (M)	46°37’50’’N, 10°19’05’’E	1800	2010 (100), 2011 (100)	2011 (10)
Albulapass (A)	46°34’55’’N, 09°48’50’’E	2250	2010 (100)	2010 (5)
Corviglia (C)	46°30’20’’N, 09°49’55’’E	2200	2011 (100)	2011 (10)

Table A2: Female reproductive success (mean ± SE) of *Gymnadenia odoratissima* plants in the four lowland and the four mountain populations and the statistics of differences between lowland populations, between mountain populations, and between the two altitudinal regions.

	Mean ± SE				Statistics	
Trait	Population 1 (*n*)	Population 2 (*n*)	Population 3 (*n*)	Population 4 (*n*)	Test statistic	*P*
Number of fruits						
2010						
Lowland populations	Döttingen (75)	Remigen (91)	Linn (99)			
	15.31 ± 1.91	4.53 ± 0.68	9.64 ± 1.01		$F_2 = 21.896$	< 0.001
Mountain populations	Schatzalp (47)	Münstertal (99)	Albulapass (85)			
	13.43 ± 1.66	13.44 ± 0.97	9.75 ± 0.96		$F_2 = 6.445$	0.002
Regions					$\chi^2_1 = 1.373$	0.242
2011						
Lowland populations	Döttingen (96)	Remigen (57)	Linn (100)	Rossweid (95)		
	11.73 ± 1.29	17.21 ± 1.52	17.09 ± 1.07	27.11 ± 1.40	$F_3 = 28.574$	< 0.001
Mountain populations	Schatzalp (94)	Münstertal (97)		Corviglia (83)		
	16.19 ± 0.86	18.82 ± 1.16		18.57 ± 1.16	$F_2 = 0.596$	0.552
Regions					$\chi^2_1 = 0.057$	0.811
Proportional female reproductive success						
2010						
Lowland populations	Döttingen (75)	Remigen (91)	Linn (99)			

	18.65 ± 2.00	10.11 ± 1.32	22.80 ± 1.99		F_2 = 15.878	< 0.001
Mountain populations	Schatzalp (47)	Münstertal (99)	Albulapass (85)			
	33.93 ± 3.54	45.73 ± 2.20	26.73 ± 2.30		F_2 = 18.626	< 0.001
Regions					χ^2_1 = 5.081	0.024
2011						
Lowland populations	Döttingen (96)	Remigen (57)	Linn (100)	Rossweid (95)		
	19.20 ± 1.89	35.71 ± 2.83	42.42 ± 2.09	55.96 ± 2.14	F_3 = 53.18	< 0.001
Mountain populations	Schatzalp (94)	Münstertal (97)		Corviglia (83)		
	48.38 ± 2.10	56.22 ± 2.58		52.26 ± 2.69	F_2 = 3.572	0.029
Regions					χ^2_1 = 3.086	0.079

Table A3: Factor loadings of display size and floral scent compounds of *Gymnadenia odoratissima* plants on principal components (PCs) using the two-year data set.

Trait	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Display size							
Plant height	0.097	-0.004	**0.857**	0.003	-0.009	-0.095	0.035
Inflorescence length	0.049	-0.020	**0.881**	0.038	0.044	-0.054	0.028
Number of flowers	0.067	-0.014	**0.822**	0.085	0.025	0.014	-0.030
Floral scent							
Aromatics							
Styrene	0.105	0.445	-0.079	-0.091	**0.447**	0.010	0.184
Benzaldehyde	**0.874**	0.110	0.067	0.289	0.089	0.033	-0.020
Benzyl alcohol	0.336	0.032	0.045	**0.845**	0.157	0.021	0.057
Phenylacetaldehyde	**0.828**	0.054	0.061	0.222	0.005	0.026	-0.065
Phenylethyl alcohol	0.363	0.039	0.061	**0.833**	0.085	0.048	0.005
Benzyl acetate	**0.903**	0.062	0.096	-0.032	0.076	0.055	0.061
1-Phenyl-1,2-propanedione	**0.782**	0.102	0.001	0.237	0.148	0.072	0.038
Phenylethylacetate	**0.858**	0.060	0.118	-0.015	0.089	0.057	0.041
1-Phenyl-2,3-butanedione	**0.851**	0.068	-0.016	0.162	-0.003	0.014	-0.006
Eugenol	**0.566**	0.088	0.055	0.442	0.093	0.033	0.294
Methyl eugenol	0.056	0.013	0.045	0.052	0.036	0.017	**0.880**
Benzyl benzoate	0.007	0.064	-0.015	0.033	0.053	-0.027	**0.871**
Terpenoids							
α-Pinene	0.254	**0.639**	0.085	-0.036	0.108	0.025	-0.027
Sabinene	0.026	**0.787**	-0.054	0.049	0.080	0.068	0.042
β-Pinene	0.041	**0.904**	-0.037	0.021	0.110	0.008	0.013
6-Methyl-5-hepten-2-one	0.103	0.141	0.063	0.164	**0.824**	0.101	-0.035
Limonene	0.025	**0.795**	0.010	0.126	0.067	0.057	0.021
Geranyl acetone	0.079	0.100	-0.025	0.075	**0.858**	0.008	0.059
Fatty acid derivatives							
(Z)-3-Hexen-1-ol	-0.017	-0.006	-0.108	0.076	0.047	**0.890**	-0.002
Heptanal	0.111	0.274	0.244	0.358	**0.430**	0.151	0.061
(Z)-3-Hexenyl acetate	0.064	0.084	-0.128	0.042	-0.013	**0.899**	-0.031
Hexyl acetate	0.249	0.129	0.217	-0.055	0.253	**0.472**	0.041

Note: For each trait, the highest loading is highlighted in bold. The PCs were extracted from a principal component analysis, which was conducted on traits standardized per population to 0 ± 1 (mean ± SD) using varimax rotation. The seven PCs with an eigenvalue > 1 explained 71.8% of the total variance. PC1 explained 21.8% of the total variance, PC2 11.6%, PC3 10.0%, PC4 9.1%, PC5 7.2%, PC6 6.5%, and PC7 5.7%.

Table A4: Factor loadings of floral signals of *Gymnadenia odoratissima* on principal components (PCs) using the 2011 data set, which included also floral color.

Trait	PC1A	PC2A	PC3A	PC4A	PC5A	PC6A	PC7A
Display size							
Plant height	0.105	-0.057	0.071	**0.839**	-0.099	0.077	-0.028
Inflorescence length	0.042	-0.021	0.083	**0.896**	-0.074	0.035	0.008
Number of flowers	0.096	0.025	0.014	**0.820**	0.048	-0.043	0.038
Floral color							
Color code	-0.070	-0.007	-0.109	-0.052	0.056	0.028	**0.554**
Floral scent							
Aromatics							
Styrene	0.106	0.429	**0.530**	-0.152	-0.013	0.193	-0.256
Benzaldehyde	**0.913**	0.055	0.168	0.058	0.014	0.005	0.119
Benzyl alcohol	0.451	0.078	0.370	0.101	-0.046	0.134	**0.619**
Phenylacetaldehyde	**0.857**	0.048	0.041	0.062	0.009	-0.048	0.098
Phenylethyl alcohol	0.457	0.092	0.254	0.086	0.012	-0.007	**0.702**
Benzyl acetate	**0.899**	0.052	0.011	0.078	0.039	0.052	-0.089
1-Phenyl-1,2-propanedione	**0.770**	0.088	0.243	0.031	0.069	0.062	0.078
Phenylethylacetate	**0.865**	0.082	0.032	0.099	0.005	-0.008	-0.066
1-Phenyl-2,3-butanedione	**0.860**	0.069	-0.021	-0.011	0.027	0.002	0.032
Eugenol	**0.674**	0.076	0.146	0.065	0.007	0.202	0.316
Methyl eugenol	0.057	0.005	0.062	0.044	0.016	**0.882**	0.028
Benzyl benzoate	0.042	0.086	0.105	0.016	0.013	**0.871**	0.060
Terpenoids							
α-Pinene	0.360	**0.636**	0.172	-0.006	0.038	-0.020	-0.060
Sabinene	0.032	**0.832**	0.140	-0.034	0.107	0.017	0.048
β-Pinene	0.062	**0.928**	0.168	-0.020	0.035	0.052	0.030
6-Methyl-5-hepten-2-one	0.086	0.240	**0.757**	0.114	0.114	-0.030	0.104
Limonene	0.014	**0.828**	0.142	0.031	0.011	0.037	0.078
Geranyl acetone	0.066	0.185	**0.783**	-0.056	0.066	0.118	-0.049
Fatty acid derivatives							
(Z)-3-Hexen-1-ol	-0.028	-0.029	0.107	-0.100	**0.894**	0.048	0.121
Heptanal	0.180	0.112	**0.745**	0.204	0.013	0.064	0.168

(Z)-3-Hexenyl acetate	0.056	0.169	-0.004	-0.072	**0.916**	-0.005	-0.004
Hexyl acetate	0.332	0.067	0.330	0.165	**0.405**	-0.032	-0.135

Note: For each trait, the highest loading is highlighted in bold. The PCs were extracted from a principal component analysis, which was conducted on traits standardized per population to 0 ± 1 (mean ± SD) using varimax rotation. The seven PCs with an eigenvalue > 1 explained 71.8% of the total variance. PC1A explained 20.4% of the total variance, PC2A 11.4%, PC3A 9.5%, PC4A 8.1%, PC5A 8.0%, PC6A 7.6%, and PC7A 6.7%.

Table A5: Differences in floral traits between lowland and mountain plants.

Traits	Principle component (PC)[a]	Mean ± SE		Statistics		
		Lowland region	Mountain region	Region[b]	Population[c]	Year[d]
Display size						
Plant height [cm]	PC3	31.85 ± 0.42	22.18 ± 0.25	780.1***	430.9***	151.1***
Inflorescence length [cm]	PC3	6.05 ± 0.10	5.37 ± 0.07	72.6***	370.2***	30.8***
Number of flowers	PC3	51.37 ± 0.84	33.90 ± 0.47	365.3***	384.7***	3.4
Floral color						
Color code		4.42 ± 0.04	1.89 ± 0.05	1677.1***	50.8***	
Floral scent [ng/l/inflorescence]						
Benzaldehyde[e]	PC1	370.01 ± 19.22	361.03 ± 20.25	0.4	306.0***	635.9***
Phenylacetaldehyde[e]	PC1	563.39 ± 31.91	428.65 ± 26.81	10.6**	294.5***	233.0***
Benzyl acetate[e]	PC1	174.93 ± 9.14	145.55 ± 8.70	9.1**	180.7***	121.4***
1-Phenyl-1,2-propanedione	PC1	1.45 ± 0.07	1.85 ± 0.09	33.5***	189.1***	325.1***
Phenylethyl acetate[e]	PC1	341.95 ± 17.40	210.20 ± 10.36	19.7***	211.2***	35.9***
1-Phenyl-2,3-butanedione[e]	PC1	19.62 ± 1.31	23.14 ± 1.84	0.2	233.2***	7.3**
Eugenol[e]	PC1	49.56 ± 2.75	14.82 ± 1.09	195.0***	256.8***	6.0*
α-Pinene	PC2	42.29 ± 2.34	55.98 ± 2.29	250.1***	336.6***	669.8***
Sabinene	PC2	74.33 ± 7.70	9.41 ± 0.76	366.2***	964.0***	356.5***
β-Pinene	PC2	21.21 ± 1.37	20.82 ± 1.42	54.5***	559.2***	643.8***
Limonene	PC2	228.36 ± 10.41	494.10 ± 55.20	195.7***	752.6***	155.3***

Benzyl alcohol	PC4	67.93 ± 4.62	41.51 ± 2.79	25.1***	453.6***	65.4***
Phenylethyl alcohol	PC4	135.82 ± 8.12	60.65 ± 5.06	85.9***	350.7***	24.2***
Styrene	PC5	29.49 ± 2.37	33.89 ± 2.33	15.6***	175.0***	5.2*
6-Methyl-5-heptene-2-one	PC5	545.36 ± 26.67	376.66 ± 17.31	24.2***	340.2***	4270.3***
Geranyl acetone	PC5	6.21 ± 0.25	5.46 ± 0.18	3.6	243.6***	134.4***
Heptanal	PC5	17.38 ± 0.60	18.81 ± 0.56	102.8***	429.4***	1602.6***
(Z)-3-Hexen-1-ol	PC6	13.06 ± 1.33	18.08 ± 2.05	53.9***	337.5***	838.7***
(Z)-3-Hexenyl acetate	PC6	193.35 ± 15.41	346.80 ± 33.19	104.9***	493.7***	687.3***
Hexyl acetate	PC6	4.32 ± 0.50	6.22 ± 0.42	141.1***	415.2***	405.2***
Methyl eugenol	PC7	1.47 ± 0.25	0.82 ± 0.22	21.6***	23.7**	16.4***
Benzyl benzoate	PC7	1.19 ± 0.21	0.74 ± 0.26	7.1**	11.4	23.3***
Total scent amount		2902.71 ± 111.38	2675.20 ± 111.19	3.0	310.2***	614.9***

Note: The last three columns refer to effects of the independent variables and show the Wald-Chi^2 values resulting from.

[a] For details on floral trait loadings on PCs, see table A3.

[b] For all traits, df = 1.

[c] For all display size and floral scent traits, df = 6; for floral color, df = 5.

[d] For all traits, df = 1.

[e] Floral scent compounds that have been shown to elicit EAD responses in *G. odoratissima* pollinators (Huber, F. K., R. Kaiser, W. Sauter, and F. P. Schiestl. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). Oecologia 142:564-575.).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table A6: Median (minimum-maximum) coefficient of variation for the three floral-signal groups of *Gymnadenia odoratissima* plants.

Coefficient of variation	Display size (*n* = 3)	Floral scent (*n* = 22)	Floral color (*n* = 1)
Within population	0.277 (0.213-0.291)[1]	1.113 (0.436-4.157)[2]	0.278[1]
Among population	0.206 (0.194-0.209)[1]	0.644 (0.352-0.943)[2]	0.091[1]

Note: Significant differences are indicated by different superscript numbers. "*n*" refers to the number of traits included in each floral signal group.

Table A7: Pollen limitation in lowland and mountain populations of *Gymnadenia odoratissima* in 2010 and 2011 assessed in a pollination experiment.

		Open-pollinated plants		Hand-pollinated plants		Mann-Whitney U test	
Population	Year	*n*	Median (minimum-maximum)	*n*	Median (minimum-maximum)	*z*	*P*
Lowland region							
Döttingen	2010	75	0.12 (0.00-0.71)	3	0.50 (0.45-0.59)	2.547	0.011
	2011	96	0.13 (0.00-0.71)	9	0.83 (0.38-1.00)	4.699	< 0.001
Remigen	2011	57	0.37 (0.00-0.77)	8	1.00 (0.67-1.00)	4.424	< 0.001
Linn	2011	100	0.44 (0.00-0.84)	7	0.87 (0.43-1.00)	3.755	< 0.001
Rossweid	2011	95	0.59 (0.00-0.86)	8	0.60 (0.40-0.90)	0.536	0.592
Mountain region							
Schatzalp	2010	47	0.33 (0.00-0.84)	5	0.60 (0.50-1.00)	2.546	0.011
	2011	94	0.50 (0.00-0.88)	10	0.78 (0.71-0.93)	4.406	< 0.001
Münstertal	2011	97	0.62 (0.00-0.98)	10	1.00 (0.75-1.00)	4.903	< 0.001
Albulapass	2010	85	0.24 (0.00-0.79)	3	0.30 (0.24-0.76)	1.176	0.240
Corviglia	2011	83	0.58 (0.00-0.90)	9	0.91 (0.56-1.00)	4.107	< 0.001

Note: Shown are sample sizes and the median (minimum-maximum) proportional female reproductive success of open-pollinated control plants and hand-pollinated plants as well as the statistics of the Mann-Whitney U tests.

Table A8: Pollinators caught and/or observed on *Gymnadenia odoratissima* inflorescences in the four lowland populations (Döttingen, Remigen, Linn, and Rossweid) and the three mountain populations (Schatzalp, Albulapass, and Corviglia).

Order	Family	Species	No. (No. caught)	No. with pollinaria	Population	Date	Time
Lepidoptera	Elachistidae	*Anchinia grisescens* FREY	4 (2)	0	Corviglia	08 Aug. 2012	17:30-21:40
		Anchinia laureolella H.-S.	3 (1)	0	Corviglia	08 Aug. 2012	17:10-18:52
	Gelechiidae	*Acompsia tripunctella* DENIS & SCHIFF.	2 (1)	0	Corviglia	03 Aug. 2012	11:35-11:36
	Zygaenidae	*Adscita geryon* HBN.	1 (1)	1	Albulapass	28 July 2010	14:00
			1 (1)	1	Corviglia	03 Aug. 2012	09:30
		Adscita spp.	3 (0)	0	Albulapass	23 July 2012	15:33-16:45
			8 (0)	6	Corviglia	03 Aug. 2012	10:17-12:00
			5 (0)	1		08 Aug. 2012	13:16-14:20
		Zygaena exulans HOCHENWARTH	1 (1)	1	Schatzalp	10 July 2012	15:30
		Zygaena transalpina ESP.	1 (1)	0	Corviglia	03 Aug. 2011	13:15
		Zygaena filipendulae L.	1 (1)	0	Corviglia	03 Aug. 2011	14:05
		Zygaena spp.	4 (0)	0	Corviglia	03 Aug. 2012	10:00-10:58
			1 (0)	0		08 Aug. 2012	12:38
	Tortricidae	*Eana argentana* CL.	1 (1)	0	Schatzalp	19 July 2011	10:00
			1 (1)	0	Corviglia	08 Aug. 2012	20:24
	Pterophoridae	*Platyptilia gonodactyla* DENIS & SCHIFF.	1 (0)	0	Schatzalp	24 July 2012	17:09
			1 (0)	0		31 July 2012	08:29
		Stenoptilia spp.	1 (1)	0	Corviglia	08 Aug. 2012	13:00

	Hellinsia osteodactyla Z.	2 (2)	2	Schatzalp	14 July 2010	18:00-18:00
	Pterophoridae spp.	1 (1)	0	Linn	14 July 2011	11:45
		1 (0)	0	Corviglia	03 Aug. 2012	10:53
		12 (0)	2		08 Aug. 2012	14:50-20:54
Pyralidae	*Endotricha flammealis* DENIS & SCHIFF.	1 (1)	0	Remigen	15 July 2011	21:00
	Oncocera semirubella SCOP.	2 (2)	2	Remigen	15 July 2011	19:00-20:15
		1 (1)	1	Linn	16 July 2011	10:30
	Pempelia palumbella DENIS & SCHIFF.	1 (1)	0	Schatzalp	14 July 2010	22:30
Crambidae	*Eudonia sudetica* Z.	3 (3)	0	Schatzalp	14 July 2010	22:00-22:30
		4 (1)	1		24 July 2012	17:16-18:10
		5 (5)	0		31 July 2012	16:17-22:10
		14 (5)	2	Corviglia	08 Aug. 2012	16:25-22:15
	Crambus lathoniellus ZNCK.	1 (1)	1	Schatzalp	24 July 2012	17:30
	Catoptria radiella HBN.	1 (1)	1	Corviglia	03 Aug. 2012	10:50
	Catoptria conchella DENIS & SCHIFF.	1 (1)	0	Schatzalp	26 July 2012	11:19
		1 (1)	0		31 July 2012	16:05
	Crambus/*Catoptria* spp.	1 (0)	0	Schatzalp	24 July 2012	10:44
		1 (0)	1	Corviglia	08 Aug. 2012	19:33
	Pyrausta despicata L.	1 (1)	1	Döttingen	06 July 2010	range 10:00-14:30
		1 (1)	1		05 July 2012	11:12

Hesperiidae	*Ochlodes sylvanus* ESP.	1 (1)	1	Döttingen	05 July 2012	12:15
		1 (1)	0		07 July 2012	16:05
		1 (1)	0	Remigen	18 July 2012	11:10
		3 (0)	0		23 July 2012	11:55-12:17
		3 (0)	0	Rossweid	09 July 2012	11:15-12:41
		3 (2)	0		13 July 2012	12:30-12:42
Pieridae	*Pieris rapae* L.	1 (1)	0	Döttingen	03 July 2012	11:20
	Pieris napi L.	1 (0)	0	Schatzalp	24 July 2012	09:45
Lycaenidae	*Lycaena tityrus* PODA.	1 (1)	0	Döttingen	03 July 2012	11:40
		1 (1)	0		07 July 2012	16:00
	Plebejus idas L.	1 (1)	1	Albulapass	28 July 2010	14:00
	Plebejus orbitulus PRUN.	1 (1)	1	Corviglia	03 Aug. 2012	11:25
	Polyommatus icarus ROTTEMBURG	2 (0)	0	Corviglia	08 Aug. 2012	11:40-11:55
	Polyommatus eros O.	1 (0)	0	Albulapass	23 July 2012	16:18
		1 (1)	0	Corviglia	03 Aug. 2012	09:40
	Polyommatus coridon PODA.	1 (1)	0	Schatzalp	21 July 2011	12:45
		2 (2)	0		26 July 2012	11:36-11:45
	Polyommatus spp.	5 (0)	0	Schatzalp	26 July 2012	10:50-11:40
		4 (0)	0		01 Aug. 2012	range 09:00-16:00
		3 (0)	0		02 Aug. 2012	16:30-18:00
		2 (0)	0		03 Aug. 2012	07:30-11:45

		1 (0)	0	Albulapass	23 July 2012	16:15
		6 (0)	0	Corviglia	03 Aug. 2012	09:35-11:50
		11 (0)	1		08 Aug. 2012	11:27-15:23
Nymphalidae	*Erebia ligea* L.	1 (1)	0	Schatzalp	26 July 2012	10:40
	Erebia pronoe ESP.	1 (1)	0	Schatzalp	21 July 2011	12:40
	Aphantopus hyperantus L.	1 (1)	0	Döttingen	03 July 2012	12:30
	Maniola jurtina L.	1 (1)	0	Linn	15 July 2011	10:00
		2 (2)	0	Döttingen	07 July 2012	15:30-16:15
		1 (0)	0	Remigen	18 July 2012	17:12
	Argynnis adippe	1 (0)	1	Remigen	18 July 2012	15:46
	DENIS & SCHIFF.					
	Euphydryas aurinia debilis ROTTEMBURG	2 (0)	1	Albulapass	23 July 2012	15:40-16:22
Geometridae	*Elophos dilucidaria*	1 (1)	0	Schatzalp	01 Aug. 2012	12:54
	DENIS & SCHIFF.					
		1 (1)	0	Corviglia	08 Aug. 2012	13:20
	Scopula incanata L.	1 (1)	0	Corviglia	08 Aug. 2012	18:15
	Scopula ternata SCHRANK	1 (1)	0	Schatzalp	14 July 2010	23:05
	Entephtia caesiata	1 (1)	1	Schatzalp	14 July 2010	23:00
	DENIS & SCHIFF.					
		1 (1)	0		31 July 2012	21:30
Noctuidae	*Phytometra viridaria* CL.	2 (1)	1	Döttingen	29 June 2012	19:20-21:00

			1 (1)	1	Remigen	12 July 2011	11:45
			2 (2)	0		15 July 2011	15:15-17:45
			3 (3)	3		07 July 2012	15:00-17:00
			1 (0)	1		18 July 2012	15:31
Diptera	Empididae	Empididae spp.	1 (1)	0	Schatzalp	15 July 2010	19:20
			1 (1)	0		19 July 2011	10:15
			1 (1)	1		21 July 2011	13:40
			1 (0)	0		24 July 2012	12:00
			9 (3)	0		26 July 2012	10:15-14:57
			1 (1)	0		31 July 2012	16:58
			1 (0)	0		09 Aug. 2012	11:15
			2 (1)	0	Corviglia	08 Aug. 2012	14:35-20:21
Coleoptera		Coleoptera sp. 1	1 (1)	1	Linn	16 July 2011	18:15
Coleoptera		Coleoptera sp. 2	1 (1)	0	Schatzalp	01 Aug. 2012	10:14

Table A9: Contribution of pollinator taxa to the differences in the pollinator communities between the lowland and the mountain region as well as between populations within altitudinal regions of *Gymnadenia odoratissima* using SIMPER (Similarity Percentages) analyses.

Pollinator taxa	Average abundance		Contribution (mean ± SD %)	Cumulative contribution [%]
	Lowland region	Mountain region		
Regions				
Lycaenidae	0.019	1.015	23.97 ± 4.70	25.43
Zygaenidae	0.000	1.039	21.21 ± 16.69	47.93
Crambidae	0.019	0.419	11.98 ± 8.77	60.64
Nymphalidae	0.070	0.351	7.38 ± 8.46	68.47
Diptera	0.000	0.168	6.94 ± 8.80	75.84
Pterophoridae	0.017	0.278	6.28 ± 6.26	82.50
Hesperiidae	0.161	0.000	4.63 ± 5.19	87.41
Noctuidae	0.107	0.000	2.84 ± 4.39	90.43
Elachistidae	0.000	0.121	2.46 ± 3.63	93.03
Geometridae	0.000	0.070	2.37 ± 2.15	95.54
Pyralidae	0.055	0.009	1.53 ± 1.53	97.17
Tortricidae	0.000	0.026	0.77 ± 0.58	97.98
Coleoptera	0.017	0.009	0.72 ± 0.80	98.75
Gelechiidae	0.000	0.035	0.70 ± 1.04	99.49
Pieridae	0.009	0.009	0.48 ± 0.55	100.00
Lowland populations	Döttingen	Remigen		
Noctuidae	0.015	0.071	28.11 ± 21.72	33.47
Hesperiidae	0.015	0.041	18.26 ± 23.25	55.20
Nymphalidae	0.022	0.020	12.40 ± 14.54	69.96
Pyralidae	0.000	0.030	8.99 ± 18.49	80.67
Crambidae	0.015	0.000	7.87 ± 11.42	90.04
Lycaenidae	0.015	0.000	5.45 ± 7.28	96.52
Pieridae	0.007	0.000	2.92 ± 6.29	100.00
	Döttingen	Linn		
Nymphalidae	0.022	0.023	18.47 ± 21.65	19.85

Pterophoridae	0.000	0.023	15.44 ± 23.58	36.45
Crambidae	0.015	0.000	10.69 ± 14.20	47.94
Pyralidae	0.000	0.023	10.42 ± 15.56	59.14
Coleoptera	0.000	0.023	10.42 ± 15.56	70.34
Noctuidae	0.015	0.000	9.23 ± 19.47	80.26
Hesperiidae	0.015	0.000	7.76 ± 10.33	88.60
Lycaenidae	0.015	0.000	6.87 ± 8.90	95.99
Pieridae	0.007	0.000	3.73 ± 7.83	100.00
	Döttingen	Rossweid		
Hesperiidae	0.015	0.185	63.70 ± 13.85	70.91
Nymphalidae	0.022	0.000	6.96 ± 9.55	78.66
Crambidae	0.015	0.000	6.21 ± 8.05	85.57
Noctuidae	0.015	0.000	5.73 ± 12.07	91.94
Lycaenidae	0.015	0.000	4.73 ± 6.13	97.21
Pieridae	0.007	0.000	2.51 ± 5.28	100.00
	Remigen	Linn		
Noctuidae	0.071	0.000	29.50 ± 22.40	31.29
Hesperiidae	0.041	0.000	16.14 ± 25.66	48.41
Nymphalidae	0.020	0.023	14.70 ± 18.73	64.01
Pyralidae	0.030	0.023	14.66 ± 18.03	79.56
Pterophoridae	0.000	0.023	11.13 ± 17.97	91.37
Coleoptera	0.000	0.023	8.14 ± 12.53	100.00
	Remigen	Rossweid		
Hesperiidae	0.041	0.185	43.97 ± 23.91	57.31
Noctuidae	0.071	0.000	20.57 ± 15.57	84.13
Pyralidae	0.030	0.000	6.94 ± 14.63	93.18
Nymphalidae	0.020	0.000	5.23 ± 11.03	100.00
	Linn	Rossweid		
Hesperiidae	0.000	0.185	67.62 ± 8.04	67.61
Pterophoridae	0.023	0.000	9.07 ± 14.04	76.68
Nymphalidae	0.023	0.000	9.07 ± 14.04	85.75
Pyralidae	0.023	0.000	7.13 ± 11.04	92.87
Coleoptera	0.023	0.000	7.13 ± 11.04	100.00

Mountain populations	Schatzalp	Albulapass		
Zygaenidae	0.002	1.000	42.67 ± 3.60	44.67
Lycaenidae	0.038	0.750	33.90 ± 9.44	80.15
Nymphalidae	0.004	0.500	13.87 ± 13.84	94.67
Crambidae	0.036	0.000	1.90 ± 3.82	96.66
Diptera	0.033	0.000	1.72 ± 3.41	98.46
Geometridae	0.009	0.000	0.49 ± 1.08	98.97
Pterophoridae	0.009	0.000	0.48 ± 1.06	99.47
Torticidae	0.002	0.000	0.14 ± 0.53	99.62
Coleoptera	0.002	0.000	0.13 ± 0.49	99.75
Pyralidae	0.002	0.000	0.12 ± 0.47	99.88
Pieridae	0.002	0.000	0.12 ± 0.46	100.00
	Schatzalp	Corviglia		
Zygaenidae	0.002	0.364	35.98 ± 22.28	38.39
Lycaenidae	0.038	0.364	20.10 ± 12.00	59.84
Crambidae	0.036	0.277	12.50 ± 13.26	73.18
Pterophoridae	0.009	0.242	8.87 ± 9.15	82.65
Diptera	0.033	0.035	5.24 ± 8.39	88.24
Elachistidae	0.000	0.121	3.79 ± 5.44	92.28
Gelechiidae	0.000	0.035	2.42 ± 3.50	94.87
Geometridae	0.009	0.035	2.15 ± 3.41	97.16
Torticidae	0.002	0.017	1.04 ± 2.84	98.28
Nymphalidae	0.004	0.000	0.67 ± 2.52	98.99
Coleoptera	0.002	0.000	0.35 ± 1.70	99.37
Pyralidae	0.002	0.000	0.31 ± 1.43	99.69
Pieridae	0.002	0.000	0.29 ± 1.32	100.00
	Albulapass	Corviglia		
Zygaenidae	1.000	0.364	20.36 ± 14.17	32.13
Lycaenidae	0.750	0.364	16.36 ± 16.86	57.94
Nymphalidae	0.500	0.000	10.64 ± 12.31	74.72
Crambidae	0.000	0.277	5.73 ± 7.97	83.76
Pterophoridae	0.000	0.242	5.04 ± 6.87	91.71
Elachistidae	0.000	0.121	2.41 ± 3.89	95.52

Gelechiidae	0.000	0.035	1.11 ± 1.88	97.28
Geometridae	0.000	0.035	0.69 ± 1.11	98.37
Diptera	0.000	0.035	0.69 ± 1.11	99.46
Torticidae	0.000	0.017	0.34 ± 0.56	100.00

Note: The taxa most important for the differences (together contributing ≥ 70% of the difference) are highlighted in bold.

Online Appendix Figure Legends

Figure A1: Percentage of plants experiencing floral herbivory (upper graphs) and differences in the mean ± SE floral herbivory (lower graphs) measured as (*A*) eaten flowers and (*B*) aphid load (scale from 1 [no aphids] to 6 [many aphids]) in the lowland and the mountain populations of *Gymnadenia odoratissima*. Sample sizes are indicated inside the top of the percentage bars. Significances of the differences between lowland populations ("D" Döttingen, "R" Remigen, "L" Linn, "RW" Rossweid), between mountain populations ("S" Schatzalp, "M" Münstertal, "A" Albulapass, "C" Corviglia), and between altitudinal regions are indicated at the top of the barplots: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $P > 0.05$.

Figure A2: Linear selection gradients β ± SE for principal components (PCs) in the lowland and mountain region in *Gymnadenia odoratissima* for the 2011 data set, which included floral color. $n_{\text{lowland}} = 312$ (four populations), $n_{\text{mountain}} = 251$ (three populations). Significances of the linear selection gradients β are indicated directly above the bars and significances of differences between altitudinal regions at the top of the graph: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. A short description of the floral signals loading primarily on each PC is given; for details, see table A4. According to variables loading primarily on PCs, PC1A corresponds to PC1 in figure 1, PC2A to PC2, PC3A to PC5, PC4A to PC3, PC5A to PC6, PC6A to PC7, and PC7A to PC4 except that floral color additionally loaded primarily on PC7A.

Figure A3: Linear selection gradients β ± SE for principal components (PCs) in lowland (left) and mountain (right) populations in *Gymnadenia odoratissima* for the 2011 data set, which included floral color. $n_{\text{Döttingen}} = 92$, $n_{\text{Remigen}} = 56$, $n_{\text{Linn}} = 92$, $n_{\text{Rossweid}} = 72$, $n_{\text{Schatzalp}} = 75$, $n_{\text{Münstertal}} = 94$, $n_{\text{Corviglia}} = 82$. Significances of the linear selection gradients β are indicated directly above the bars and significances of differences among populations at the top of the graphs: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. A short description of the floral signals loading primarily on each PC is given; for details, see table A4. According to variables loading primarily on PCs, PC1A corresponds to PC1 in figure 2, PC2A to PC2, PC3A to PC5, PC4A to PC3, PC5A to PC6, PC6A to PC7, and PC7A to PC4 except that floral color additionally loaded primarily on PC7A.

Figure A4: Comparison of the trait-value distribution of the principle components used for selection analysis in lowland (grey bars) and mountain (white bars) regions. The distributions of the traits are compared with superimposed normal distributions.

Online Appendix Figures

Figure A1

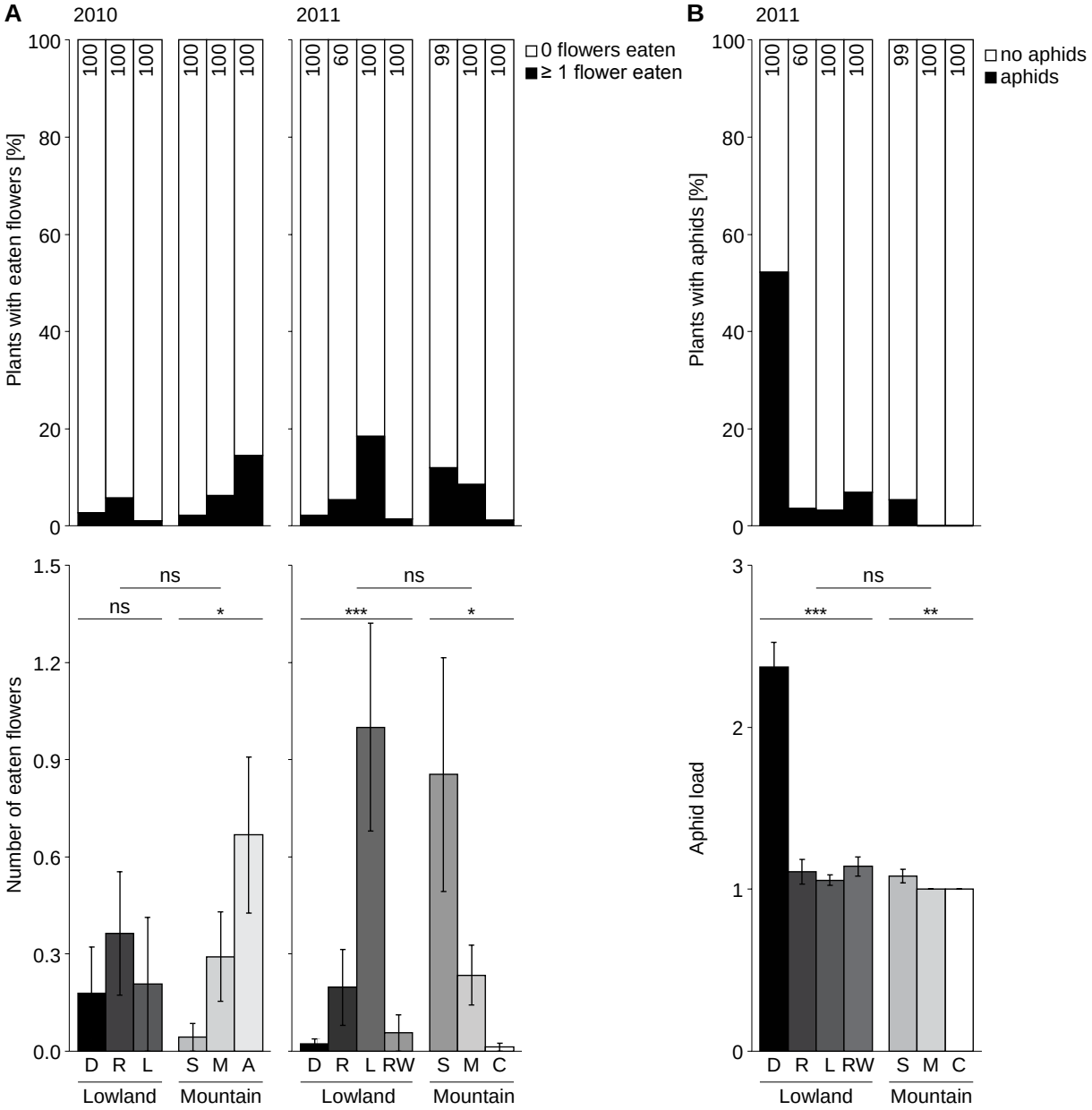


Figure A2

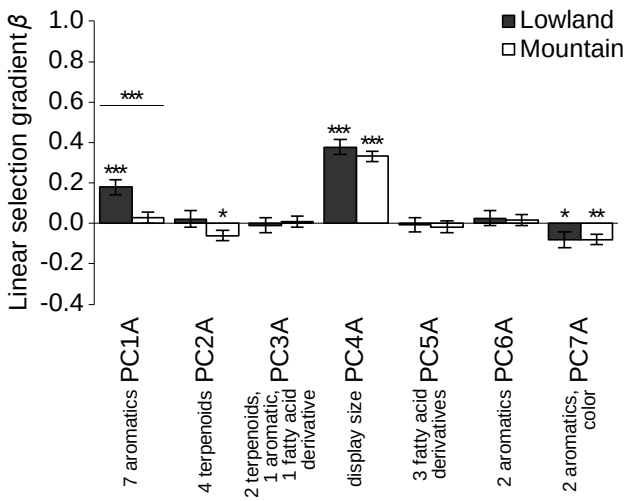

Linear selection gradient β
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Lowland
Mountain
***
***
***
***
*
*
**
PC1A
7 aromatics
PC2A
4 terpenoids
PC3A
2 terpenoids, 1 aromatic, 1 fatty acid derivative
PC4A
display size
PC5A
3 fatty acid derivatives
PC6A
2 aromatics
PC7A
2 aromatics, color

Figure A3

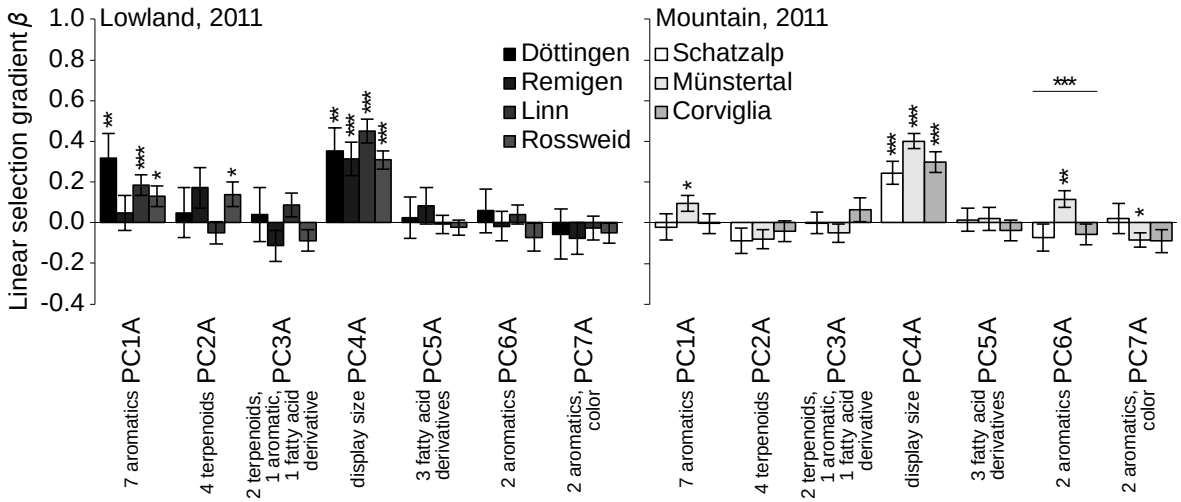
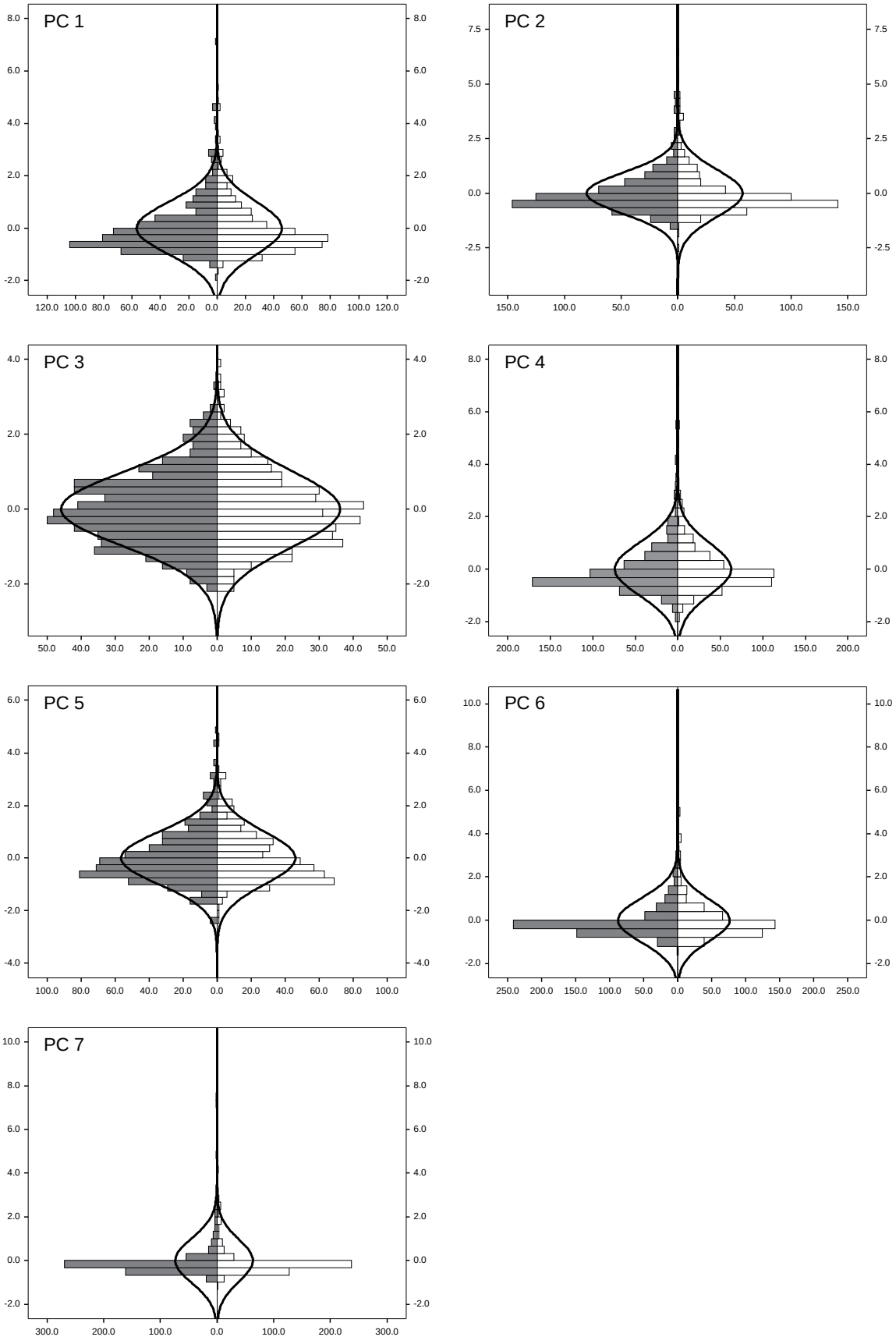

Linear selection gradient β
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Lowland, 2011
Döttingen
Remigen
Linn
Rossweid
Mountain, 2011
Schatzalp
Münstertal
Corviglia
***
7 aromatics PC1A
4 terpenoids PC2A
2 terpenoids, 1 aromatic, 1 fatty acid derivative PC3A
display size PC4A
3 fatty acid derivatives PC5A
2 aromatics PC6A
2 aromatics, color PC7A

Figure A4



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Tables

Table 1: Statistics of a linear mixed model testing for differences in directional selection on principal components (PCs) between altitudinal regions and years.

Principle components (traits with highest loadings)		PC x Region		PC x Year	
		χ^2_1	*P*	χ^2_1	*P*
PC1	(7 aromatics: benzaldehyde, phenylacetaldehyde, benzyl acetate, 1-phenyl-1,2-propanedione, phenylethylacetate, 1-phenyl-2,3-butanedione, eugenol)	13.757	< 0.001	3.816	0.051
PC2	(4 terpenoids: α-pinene, sabinene, β-pinene, limonene)	5.450	0.019	3.615	0.057
PC3	(plant height, inflorescence length, number of flowers)	1.744	0.187	14.764	< 0.001
PC4	(2 aromatics: benzyl alcohol, phenylethyl alcohol)	2.930	0.087	2.867	0.090
PC5	(1 aromatic: styrene; 2 terpenoids: 6-methyl-5-hepten-2-one, geranyl acetone; 1 fatty acid derivative: heptanal)	1.927	0.165	5.117	0.024
PC6	(3 fatty acid derivatives: (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate, hexyl acetate)	0.462	0.497	0.293	0.589
PC7	(2 aromatics: methyl eugenol, benzyl benzoate)	1.683	0.195	0.030	0.863

Note: Floral signals exhibiting highest loadings on each PC are listed in brackets. For more details, see table A3.

Table 2: Statistics of a linear model testing for differences in directional selection on principal components (PCs) between populations and years in the lowland and the mountain regions.

	Lowland				Mountain			
	PC x Population		PC x Year		PC x Population		PC x Year	
PC	F_3	P	F_1	P	F_3	P	F_1	P
PC1	0.037	0.772	1.383	0.240	2.164	0.092	0.398	0.528
PC2	1.819	0.143	1.271	0.260	0.390	0.761	0.352	0.554
PC3	0.225	0.879	5.547	0.019	3.024	0.029	1.821	0.178
PC4	0.492	0.688	0.190	0.664	0.735	0.531	3.133	0.077
PC5	0.361	0.782	3.583	0.059	2.329	0.074	0.040	0.841
PC6	1.157	0.326	0.001	0.978	0.425	0.735	1.588	0.208
PC7	0.278	0.841	0.027	0.868	6.690	< 0.001	7.389	0.007

Note: For a list of floral signals exhibiting highest loadings on each PC, see table 1. For details, see table A3.

Figure Legends

Figure 1: Linear selection gradients $\beta \pm$ SE for principal components (PCs) in the lowland and mountain regions in *Gymnadenia odoratissima*. 2010: $n_{\text{lowland}} = 253$ (three populations), $n_{\text{mountain}} = 212$ (three populations); 2011: $n_{\text{lowland}} = 312$ (four populations), $n_{\text{mountain}} = 251$ (three populations). Significances of linear selection gradients β are indicated above the bars: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. PCs with significant differences between regions but not between years (table 1; indicating consistent spatial differences in selection) are marked with red dotted quadrangles. A short description of the floral signals loading primarily on each PC is provided; for details, see table A3.

Figure 2: Linear selection gradients $\beta \pm$ SE for principal components (PCs) in lowland (left) and mountain (right) populations in *Gymnadenia odoratissima*. 2010: $n_{\text{Döttingen}} = 73$, $n_{\text{Remigen}} = 88$, $n_{\text{Linn}} = 92$, $n_{\text{Schatzalp}} = 47$, $n_{\text{Münstertal}} = 96$, $n_{\text{Albulapass}} = 69$; 2011: $n_{\text{Döttingen}} = 92$, $n_{\text{Remigen}} = 56$, $n_{\text{Linn}} = 92$, $n_{\text{Rossweid}} = 72$, $n_{\text{Schatzalp}} = 75$, $n_{\text{Münstertal}} = 94$, $n_{\text{Corviglia}} = 82$. Significances of the linear selection gradients β are indicated above the bars: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. A short description of the floral signals loading primarily on each PC is provided; for details, see table A3.

Figure 3: Correlogram of among-population Euclidean distance in floral signals that exhibited the highest loadings on the principal components (PCs) used in the selection analysis plotted by the among-population Euclidean distance in selection gradients on these PCs for (*A*) floral scent PC1, (*B*) floral scent PC2, (*C*) display size PC3, (*D*) floral scent PC4, (*E*) floral scent PC5, (*F*) floral scent PC6, and (*G*) floral scent PC7. Mantel test statistics are provided (1000 permutations in all tests); significant results ($P < 0.05$) are highlighted in bold. $n = 5$ populations (three lowland and two mountain populations). For details on PCs, see table 1 and table A3.

Figure 4: Differences in pollinator community composition in lowland and mountain populations of *Gymnadenia odoratissima*. (*A*) Non-metric multidimensional scaling (NMDS) of pollinator community composition quantified per date and population in four lowland populations (“D” Döttingen, “R” Remigen, “L” Linn, “RW” Rossweid) and three mountain populations (“S” Schatzalp, “A” Albulapass, “C” Corviglia). Pollinators shown to the left of

the plot represent the five taxa (“1” Lycaenidae, “2” Zygaenidae, “3” Crambidae, “4” Nymphalidae, “5” Diptera), which contributed most to the community differences between the lowland and mountain region and were all more common in the mountain region. Boxplots (median, quartiles, minimal, and maximal value) of (*B*) the visitation rate (number of pollinators per hour) and (*C*) pollinator richness (number of pollinator families/orders per hour) compared between the lowland (L) and mountain (M) region (left) and between the four lowland and between the three mountain populations (right). Significant differences are indicated at the top of the graphs: $*P < 0.05$.

Figures

Figure 1

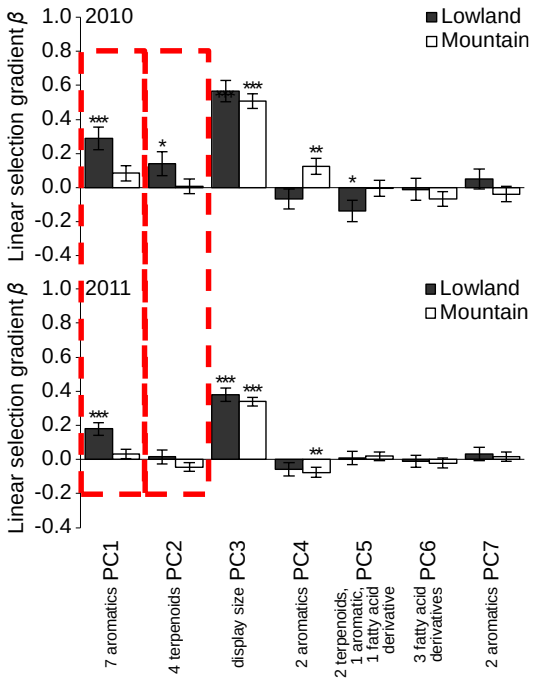


Figure 2

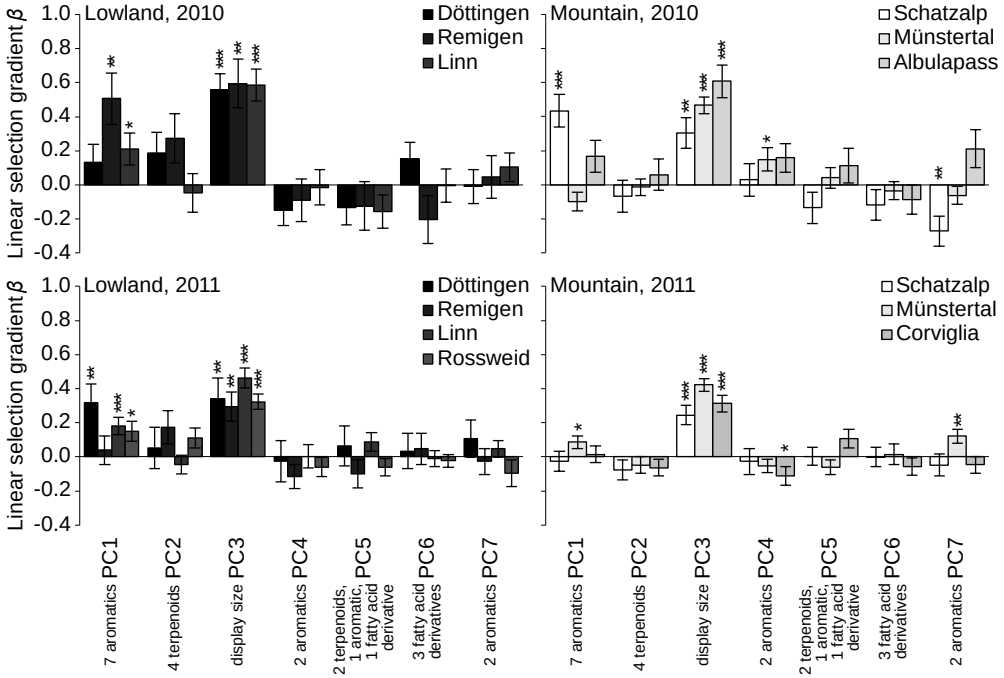

Linear selection gradient β
Lowland, 2010
Döttingen
Remigen
Linn
Mountain, 2010
Schatzalp
Münstertal
Albulapass
Lowland, 2011
Rossweid
Mountain, 2011
Corviglia
7 aromatics PC1
4 terpenoids PC2
display size PC3
2 aromatics PC4
2 terpenoids, 1 aromatic, 1 fatty acid derivative PC5
3 fatty acid derivatives PC6
2 aromatics PC7

Figure 3

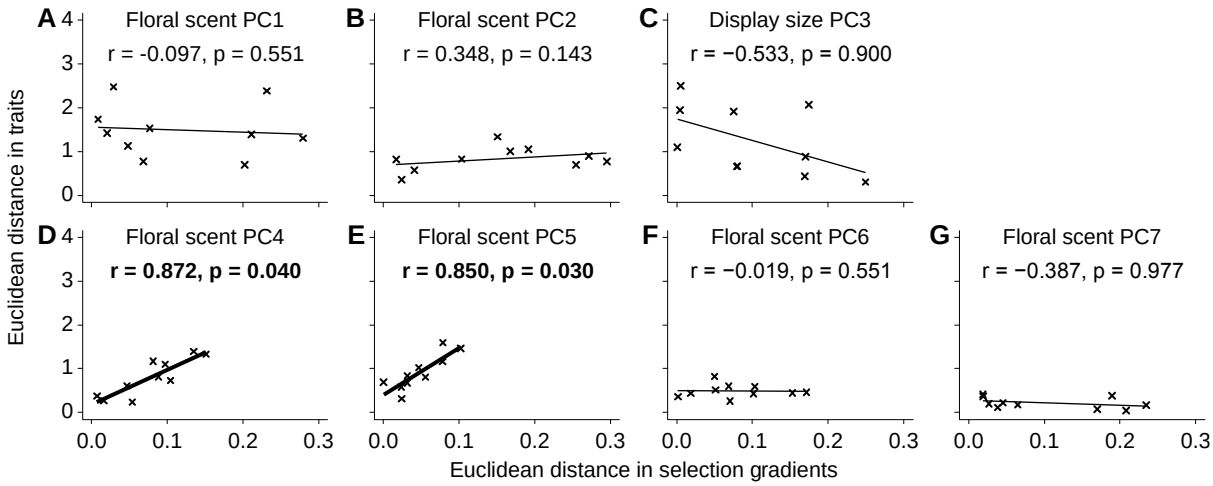
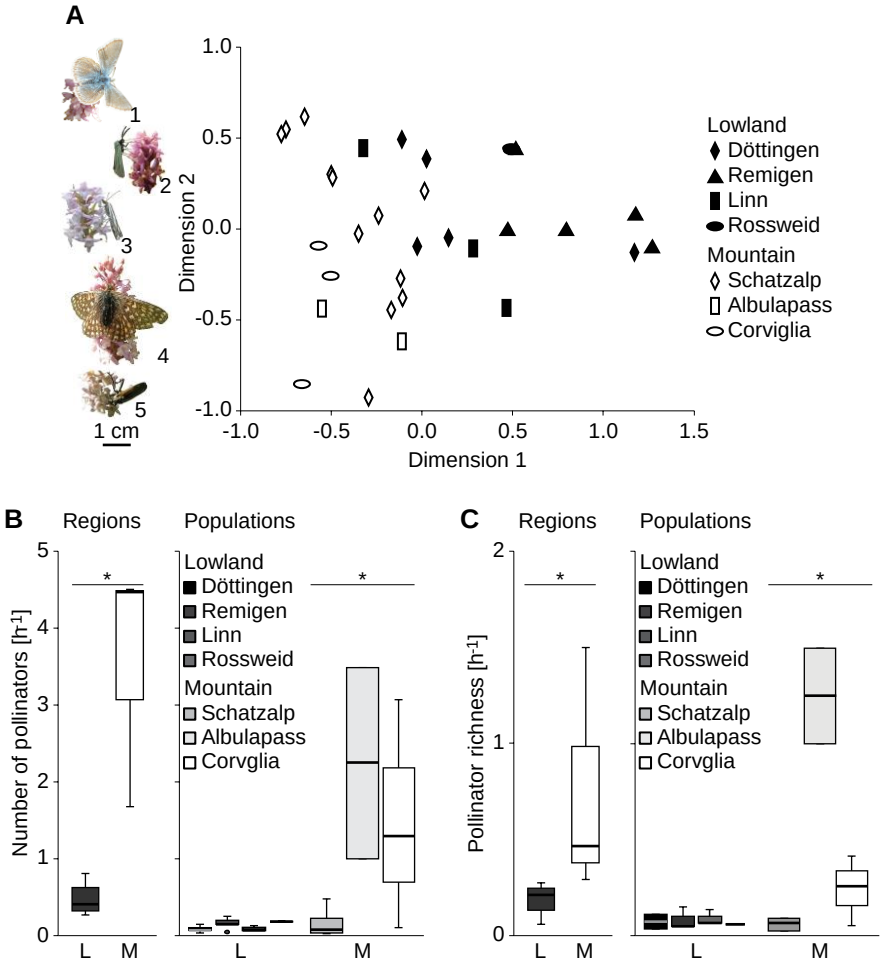

A
Floral scent PC1
r = -0.097, p = 0.551
B
Floral scent PC2
r = 0.348, p = 0.143
C
Display size PC3
r = −0.533, p = 0.900
D
Floral scent PC4
r = 0.872, p = 0.040
E
Floral scent PC5
r = 0.850, p = 0.030
F
Floral scent PC6
r = −0.019, p = 0.551
G
Floral scent PC7
r = −0.387, p = 0.977
Euclidean distance in traits
Euclidean distance in selection gradients

Figure 4


A
1
2
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1 cm
Dimension 2
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1.0
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1.0
1.5
Lowland
Döttingen
Remigen
Linn
Rossweid
Mountain
Schatzalp
Albulapass
Corvglia
B
Regions
Populations
Number of pollinators [h-1]
C
Regions
Populations
Pollinator richness [h-1]
L
M
*

Chapter 3.

FLORAL ADAPTATION TO LOCAL POLLINATOR GUILDS IN A TERRESTRIAL ORCHID

M. Sun, K. Gross and F. P. Schiestl

Part of a special issue on pollinator-driven speciation
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Author contributions
MS and FPS designed the experiment, KG also helped with the design
MS performed the majority of the field experiments, KG also helped in the field
MS performed the majority of the data analysis, KG performed the scent analysis
MS wrote the paper, contributions were made by FPS and KG

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PART OF A SPECIAL ISSUE ON POLLINATOR-DRIVEN SPECIATION

Floral adaptation to local pollinator guilds in a terrestrial orchid

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• *Background and Aims* Studies of local floral adaptation in response to geographically divergent pollinators are essential for understanding floral evolution. This study investigated local pollinator adaptation and variation in floral traits in the rewarding orchid *Gymnadenia odoratissima*, which spans a large altitudinal gradient and thus may depend on different pollinator guilds along this gradient.
• *Methods* Pollinator communities were assessed and reciprocal transfer experiments were performed between lowland and mountain populations. Differences in floral traits were characterized by measuring floral morphology traits, scent composition, colour and nectar sugar content in lowland and mountain populations.
• *Key Results* The composition of pollinator communities differed considerably between lowland and mountain populations; flies were only found as pollinators in mountain populations. The reciprocal transfer experiments showed that when lowland plants were transferred to mountain habitats, their reproductive success did not change significantly. However, when mountain plants were moved to the lowlands, their reproductive success decreased significantly. Transfers between populations of the same altitude did not lead to significant changes in reproductive success, disproving the potential for population-specific adaptations. Flower size of lowland plants was greater than for mountain flowers. Lowland plants also had significantly higher relative amounts of aromatic floral volatiles, while the mountain plants had higher relative amounts of other floral volatiles. The floral colour of mountain flowers was significantly lighter compared with the lowland flowers.
• *Conclusions* Local pollinator adaptation through pollinator attraction was shown in the mountain populations, possibly due to adaptation to pollinating flies. The mountain plants were also observed to receive pollination from a greater diversity of pollinators than the lowland plants. The different floral phenotypes of the altitudinal regions are likely to be the consequence of adaptations to local pollinator guilds.

Key words: Local adaptation, pollination, floral evolution, geographical variation, floral morphology, floral scent, VOC, floral colour, pollinator assemblages, pollinator adaptation, Diptera, Orchidaceae, speciation.

INTRODUCTION

The adaptation of plants to different pollinators is widely regarded as a key mechanism promoting the diversification and speciation of animal-pollinated angiosperms (Grant and Grant, 1965; Stebbins, 1970; Faegri and van der Pijl, 1979; Schluter, 2000; Johnson, 2006; Schiestl and Schlüter, 2009; Schiestl, 2012). Several lines of evidence support the link between animal pollinators and angiosperm diversification. These include the sudden and broad diversification of animal-pollinated plant lineages (Eriksson and Bremer, 1992; Ricklefs and Renner, 1994; Dodd *et al.*, 1999), strong selection exerted on floral traits by pollinators (e.g. Galen, 1989; Campbell *et al.*, 1997; Schiestl and Johnson, 2013) and floral phenotype associations with particular pollinator groups (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003; Fenster *et al.*, 2004; Willmer, 2011; Schiestl and Dötterl, 2012).

The first conceptual model of pollinator-driven speciation was developed by Grant and Grant (1965), who noted in a study of *Gilia leptantha* (Polemoniaceae) that the floral trait variation across a geographical range appeared to have derived from a pollinator-shift between bees and bee-flies. Stebbins (1970) expanded on the concept that divergence in floral form is often attributed to variation in geographical pollinator mosaics and adaptation to the most effective pollinator. As different pollinators vary in functional morphology, foraging behaviour, thermal biology, nutritional requirements and innate floral preferences, the geographical variability in pollinator composition could result in divergent selection pressures on floral traits between intraspecific populations. Selection mosaics on floral traits that enhance reproductive success will induce the evolution of locally adapted variants of a species. Ultimately, if the different pollination 'ecotypes' were to arrive into secondary contact, the pollinator preferences could conceivably prevent any intercrossing.

Although the Grant-Stebbins model (Johnson, 2006) is the basis for allopatric and parapatric divergence in pollination systems, studies of the role of geographical variation in pollinators and intraspecific floral adaptation to local pollinators in floral diversification remain relatively sparse (as reviewed by Coyne and Orr, 2004; Herrera *et al.*, 2006; Johnson, 2006). Recent approaches to research on the adaptive origin of floral diversity have focused on the correlation between floral diversification and pollination in the light of phylogenetics (e.g. Hapeman and Inoue, 1997; Graham and Barrett, 2004; Patterson and Givnish, 2004; van der Niet and Johnson, 2012), or on pollinator-mediated phenotypic selection on floral traits within a single natural population (e.g. Campbell *et al.*, 1991; Maad, 2000; Schiestl *et al.*, 2011; Schäffler *et al.*, 2012) or under artificial settings (e.g. Herrera, 2001; Aigner, 2004; Castellanos *et al.*, 2004).

Following the Grant–Stebbins model, a few subsequent studies presented clear connections between different pollinator assemblages and floral trait variation (e.g. Robertson and Wyatt, 1990; Johnson and Steiner, 1997; Moeller, 2005; Nattero and Cocucci, 2007; Anderson and Johnson, 2008; van der Niet *et al.*, 2014). For example, the study by Johnson and Steiner (1997) illustrated pollinator adaptation of spur length in the South African orchid *Disa draconis* complex to short- and long-tongued flies. Floral morphology diverged as plants became locally adapted to variation in the proboscis length, behaviour, colour preferences and flight period of the spatially separated fly species. Schlumpberger *et al.* (2009) documented a notable example of prominent variation in corolla lengths, nectar production and anthesis in the South American cactus species *Echinopsis ancistrophora*. Flowers with short corolla have morning anthesis and low nectar production, while longer corolla flowers have anthesis at dusk and abundant nectar. Populations with the longest corolla are also where sphingid moth pollination predominantly occurs, and pollination by solitary bees dominated the remaining populations with shorter corolla.

Most of these studies indicate local pollinator adaptation through correlational evidence between floral traits and pollinator differences. A further step is necessary to confirm that these differences in floral traits are pollinator-driven, and would act as a barrier to or reduce gene flow when the 'ecotypes' come into contact. In this study, we address this step through a reciprocal transfer experiment using the orchid species *Gymnadenia odoratissima*. Orchids are one of the most diverse plant families, with pollinator interactions considered to be the primary driving force of their diversification (Dressler, 1993; Schiestl and Schlüter, 2009; Harder and Johnson, 2009; Schiestl, 2012). Thus, orchids represent a significant model system for the investigation of pollinator adaptation. *G. odoratissima* is a nectar-rewarding species found throughout Europe, and is abundant in many calcareous regions of Switzerland where the study was conducted. The plant can inhabit lowland forests at around 500 m above sea level (m a.s.l) to subalpine meadows at up to 2600 m a.s.l. Thus, due to the large span in altitudinal gradient, it is likely that the pollinator assemblages differ in composition in lowland and mountain populations. It is known from previous studies that the pollinators of *G. odoratissima* are primarily Lepidoptera species (van der Cingel, 1995; Vöth, 2000; Huber *et al.*, 2005, and references therein), although the qualitative and quantitative variation in pollinator communities between the lowlands and mountains has not yet been defined. We characterized the pollinator community composition in the plant populations of the lowlands and mountains, and assessed any qualitative and quantitative differences between the pollinator communities. To investigate the existence of local pollinator adaptation, reciprocal transfers of cut plants were performed between lowland and mountain populations, and their pollination success compared with cut local plants was quantified. We predicted that if there is evidence of local adaptation, it would have resulted from spatially divergent evolution of floral traits. It has been shown that these traits may include display size (e.g. Galen, 1989), corolla dimensions (e.g. Nattero and Cocucci, 2007; Medel *et al.*, 2007; Anderson and Johnson, 2008; Gómez *et al.*, 2008; Martén-Rodríguez *et al.*, 2011; van der Niet *et al.*, 2014), spur length (e.g. Robertson and Wyatt, 1990; Johnson and Steiner, 1997; Anderson and Johnson, 2009; Peter and Johnson, 2014), floral scent (Mant *et al.*, 2005; Anderson *et al.*, 2009; Schiestl *et al.*, 2011; Parachnowitsch *et al.*, 2012; Peter and Johnson, 2014; van der Niet *et al.*, 2014), floral colour (e.g. Streisfeld and Kohn, 2007; Newman *et al.*, 2012) and nectar properties (e.g. Johnson and Nicolson, 2008; Schlumpberger *et al.*, 2009). Thus, we address the following questions: (1) Are there differences in the pollinator composition between lowland and mountain populations? (2) Do plants achieve lower reproductive success when transferred to a different altitudinal region compared with the local plants? (3) Are there differences in floral morphology, scent composition, colour and nectar sugar concentration between plants from the lowlands and mountains?

MATERIALS AND METHODS

Study species

Gymnadenia odoratissima (L.) L.C.M. Richard (Orchidaceae) is a terrestrial orchid species found in temperate and mountainous regions of Europe. The species has a flowering period generally from June to mid-August, with an overlap in flowering time between the lowland and mountain populations of approximately 3 weeks (M. Sun, pers. obs.). The plant inflorescences have between 10 and 100 flowers, with each flower producing nectar contained in a floral spur as food reward for pollinators. The pollination system is functionally specialized with visitations from diurnal and nocturnal Lepidoptera species (van der Cingel, 1995; Vöth, 2000; Huber *et al.*, 2005, and references therein). The flowers have colours ranging from deep pink to white, and emit strong floral scent during both the day and the night (Huber *et al.*, 2005).

Plant populations

Twelve populations (six lowland and six mountain populations) of *G. odoratissima* within Switzerland were sampled from June to mid-August between 2010 and 2012. Details of the geographical locations and the years when pollinator communities and floral traits (including sample sizes) were assessed, and transfer experiments were conducted are presented in Table S1 (Supplementary Information).

Pollinator observations

During the flowering period, pollinating insects were observed and caught from inflorescences of naturally growing plants throughout the day and evening. Insects observed (1) to probe the floral spur and feed from the nectar, (2) to have obtained pollinia or (3) to possess pollinia were classified as pollinators. These insects were caught using hand nets and individually stored in a −20 °C freezer. Commonly observed insect species, of which it was certain that the species had previously been caught, were recorded as observed but not caught. Pollinators observed from 0601 h to 1800 h were categorized as diurnal pollinators, and those observed from 1801 h to 0600 h were categorized as nocturnal. A total sampling time of 85·25 h (63·75 h during the day, 21·50 h during the night) was spent in the lowland populations, and 80·00 h (61·75 h during the day,

18·25 h during the night) in the mountain populations, calculated to the nearest 0·25 h. The number of pollinator observation hours was also used to calculate the pollinator visitation rate (number of pollinator observations per hour).

Transfer experiments

Two types of transfer experiments were conducted: vertical transfers and horizontal transfers. The vertical transfers consisted of bidirectional transfers of plants from lowland populations to mountain populations, and vice versa. The horizontal transfers were bidirectional transfers of plants between populations of the same altitude, i.e. between lowland populations and between mountain populations, as a control to test for population-specific effects on pollination success. Pollinia were removed from the experimental flowers to prevent gene pool contamination of the local populations. For each individual, any previously pollinated flowers were removed from the inflorescence, as well as any buds.

For the vertical transfers, a population of *G. odoratissima* at each of the two altitudinal levels was selected. In each population, 30 plants were randomly selected and cut at the stem at ground level. Within the 30 plants, 15 were placed in the population from which they were collected, referred to as 'local' individuals, and 15 were transferred to a population of the other altitudinal level, referred to as 'transferred' individuals. The 'local' individuals were used for comparison with the 'transferred' individuals from the other altitudinal level. In the lowland populations, the 15 'transferred' individuals were moved to the mountain population, and in the mountain populations, 15 'transferred' individuals were moved to the lowland population. The 'transferred' individuals were transported in plastic containers containing water and kept shaded throughout the transportation process. In each population, a series of 15 plots were set up along a transect, with each plot consisting of one 'local' and one 'transferred' individual. Each individual was placed in a 15-mL Falcon tube (BD, Franklin Lakes, NJ, USA) containing water, and set into the ground. The two individuals within a plot were placed approx. 20 cm apart, while the distances between the plots were approx. 2–5 m. It was ensured that the plots were at least 0·5 m from a natural neighbouring plant. This method of plant treatment does not negatively affect plant growth, as the plants were observed to develop, flower and set fruit under these conditions.

Horizontal transfers were performed in the same way as the vertical transfers, except that the 'transferred' individuals of a population were moved to another population of the same altitudinal level. The pairs of populations used in the vertical and horizontal transfers are listed in Table S2.

After a period of three weeks, all plots were collected. For each individual the number of pollinated flowers, the number of fruit capsules formed and the total number of intact flowers on the inflorescence during flowering were counted. From this, the proportionate female reproductive success (R_f) was determined for each individual using the following formula:

$R_f = (F_p + S)/F_i$

F_p is the total number of pollinated flowers, S is the total number of flowers that set fruits (fruit set) and F_i is the total number of flowers on the inflorescence. Both F_p and S were obtained to quantify female reproductive success, as the flowers on an inflorescence were at different developmental stages during plot collection. To ensure that pollinated flowers set fruit and thus can be used as a reliable measure of female reproductive success, a series of hand-pollination experiments were performed on 20 individuals in the lowland population 'Döttingen' and the mountain population 'Münstertal'. Fine-mesh wire cages were placed over each individual prior to plant flowering to exclude any pollinator visitations. During flowering, five flowers per individual were marked with coloured thread and hand-pollinated with one to two pairs of pollinia using wooden toothpicks. After three weeks, the pollinated flowers were examined for fruit capsule development.

Floral phenotype survey

Floral morphology measurement. We measured the length of inflorescences to the nearest centimetre (Fig. 1, left) by calculating the difference between plant height and stem length. Subsequently, two flowers per individual were sampled: a higher and a lower flower on the inflorescence. Flowers were stored in 2-mL Eppendorf tubes (Safe-Lock Tubes; Eppendorf AG, Hamburg, Germany) containing 70 % ethanol. In the lab, the individual flowers were placed in a clear Petri dish, and thinly immersed in a few drops of ethanol. The flowers were carefully spread out and flattened into position, facing down such that the spur and all the dimensions of the petals and sepals were entirely visible and fully extended. Photos were taken of each flower using a digital SLR camera (Nikon D90 D-SLR; Nikon Corporation, Tokyo, Japan) fitted with a 105-mm F/2·8D lens (AF-S VR Micro-Nikkor; Nikon Corporation), and attached to a fixed tripod.

Each photo was analysed using the image processing and analysis program ImageJ (http://rsbweb.nih.gov/ij/), with each floral image measurement calibrated to a 5-cm scale included. The floral traits numbered from 1 to 10 (Fig. 1, right) were measured for each flower. Flower shape (traits 1 and 2) and area (9), labellum size and shape (3, 4, 6, 7 and 10) and inflorescence size comprise the display signals for pollinator attraction, while spur length (8) affects nectar accessibility for potential pollinators. The mean value for each trait was calculated between the two flowers of each individual. The floral traits were also standardized to inflorescence size to test for any effects of resource limitation and trade-offs.

Floral scent collection and identification. Scent collection was performed during the day between 0800 and 1700 h, within the flowering period. The entire inflorescence of each individual was enclosed in oven bags (Nalophan; Kalle UK Ltd, Witham, UK) and sealed at the ends with twist close wires. Air was extracted from the bags using a battery-operated pump (PAS-500 personal air sampler Spectrex; Redwood city, CA, USA) for 30 min at a rate of 150 mL min^{-1}, through fine glass tubes containing approx. 20 mg of Tenax TA (80/100 mesh; Supelco, Bellefonte, PA, USA). In each population, the scent of the surrounding air was sampled under the same scent collection parameters, as a control. The glass tubes were sealed, transported to the lab and stored in a −25 °C freezer.

Analysis of the floral scent bouquet was conducted using gas chromatography with mass selective detection (GC-MS). Each glass tube was loaded and injected into the chromatograph

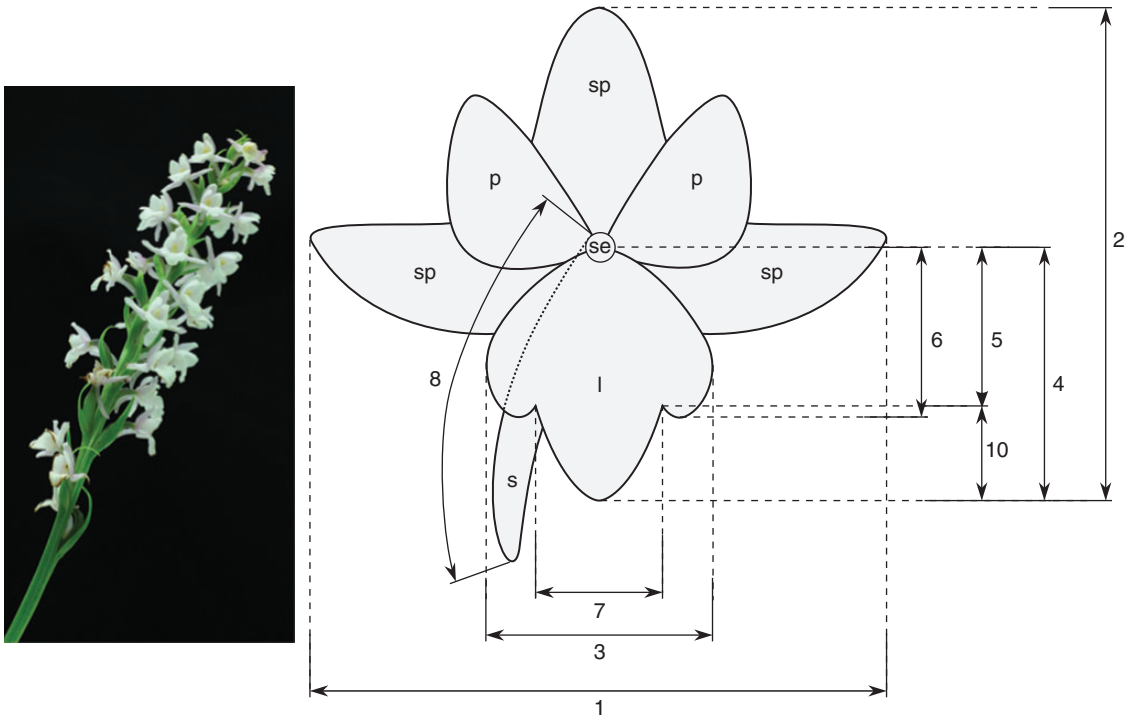

FIG. 1. *Gymnadenia odoratissima* inflorescence (left) and a diagrammatic flower (right) with morphological traits 1–10 indicated. Flower morphological traits are 1: flower width, 2: flower height, 3: labellum width, 4: labellum height, 5: spur entrance to height of interlobe, 6: side-lobe length, 7: interlobe distance, 8: spur length, 9: flower area (not shown, calculated by ImageJ from manually tracing the flower outline), 10: lobe length. Other floral traits indicated are p: petals, l: labellum, sp: sepals, s: spur, se: spur entrance.

(Agilent 6890 N) using a Gerstel thermal desorption system (TDS3, Gerstel, Mühlheim, Germany) with cold injection system (CIS; CIS4; Gerstel). For thermal desorption, the TDS was heated from 30 to 240 °C at a rate of 60 °C min^{-1} and held at the final temperature for 5 min. During the collecting of eluting compounds from the TDS, the CIS was set to –150 °C. For injection, the CIS was then heated to 250 °C at a rate of 12 °C s^{-1} and this temperature was held for 3 min. The gas chromatograph was equipped with an HP-5MS column (0·25 μm i.d., 0·32 mm film thickness, 30 m length), and helium was used as the carrier gas at 1·9 mL min^{-1} flow rate. Compound identification and quantification were achieved using a mass selective detector (Agilent MSD 5975). Chromatograms were analysed using the program ChemStation (G1701EA E.02·02 MSD Productivity ChemStation Software, Agilent Technologies, Germany). Preliminary identification of volatiles was done using the NIST spectral database implemented in the ChemStation program. Subsequently, retention times and mass spectrograms of all floral volatiles were compared with those of synthetic reference compounds. For quantification, calibration curves for qualifier ions were established for all compounds. To calculate the absolute amounts of floral volatiles, the peak areas of qualifier ions were converted into nanograms using the calibration curves. As the ChemStation program did not always correctly identify the peaks, all samples and compounds were manually double-checked and, if necessary, integrated manually. All absolute amounts were calculated as ng L^{-1} of sampled air. To exclude compounds produced in only trace amounts, a mean threshold of 0·5 ng L^{-1} air sampled per inflorescence was imposed, of which 22 compounds (for the list of compounds and their IUPAC name see Table S5) from the scent profile exceeded. We calculated the relative amounts for each of the 22 compounds separately by dividing the absolute amount of an individual compound by the sum of the absolute amounts of all compounds.

Floral colour measurement. Two flowers from each individual were sampled: one from the top and one from the lower part of the inflorescence. Each flower was wrapped in damp tissue paper, stored in a 1·5-mL Eppendorf tube (Safe-Lock Tubes) and kept in a 4 °C fridge until analysis. Flower labellum colour was measured as percentage reflectance using a AvaSpec-2048 Fibre Optic Spectrometer (Avantes B.V., Eerbeek, the Netherlands) and a AvaLight-XE xenon pulsed light source (Avantes B.V.). The fibre optic probe (Avantes B.V.) was held at a fixed distance and angle from the labellum using an enclosed fibre optic holder. The measurements were calibrated with a 98 % reflective polytetrafluoroethylene (PTFE) white and black reference tile (Avantes B.V.) at the beginning of each session of measurements. The reflectance spectra were expressed as a percentage of reflected light in relation to the white reference tile, with wavelengths between 350 nm (little to no reflectance was detected up to then) and 700 nm considered. All the equipment was connected to a laptop equipped with the data collection software AvaSoft 7·3 (AvaSoft-Basic, Avantes B.V.). Each reflectance spectrum was composed of 1206 percentage reflectance data points taken at 0·597-nm intervals. The mean percentage reflectance value at each reflectance interval was calculated between the two flowers of each individual.

Nectar sugar concentration measurement. All plants were placed under fine-mesh wire cages prior to flowering to exclude pollinators from influencing nectar volume and concentration. On the onset of anthesis, nectar was extracted from two flowers per inflorescence: from the uppermost and lowermost open flower. To measure the nectar sugar content, the spur was cut off from the flower as close to the spur entrance as possible using a pair of fine scissors. The nectar was carefully compressed out of the spur directly onto the optical glass of a hand-held refractometer (Eclipse 45-81, Bellingham & Stanley Ltd, Tubridge Wells, UK; 0–50 ° Brix units), and the sugar percentage (sucrose equivalent %) was read. The sugar percentage measurements from the two flowers of each individual were taken between 1100 and 1500 h for 20 marked individuals. This was done on the same individuals on two separate days in each population. The sugar percentage of each individual was determined from an average of the lower- and uppermost flower measurements for both days. To observe whether there were any effects of temperature and humidity on sugar concentration, for each sampled individual temperature and humidity data corresponding to the time of each measurement were taken from records at the nearest respective weather station: Beznau KKW (3·9 km from Döttingen), Ueken (4·7 km from Linn), Sta. Maria/Val Müstair (8·4 km from Münstertal) and Davos (1·1 km from Schatzalp).

Data analysis

To determine whether there was a significant difference in female reproductive success between 'local' and 'transferred' individuals, and whether the difference was affected by the altitude at which the population was situated, multiple logistic regressions were conducted. The proportionate female reproductive success was transformed into a binomial dataset (1 = pollinated, 0 = unpollinated) on an individual flower level for the conditional logistic (*clogistic*) model, to derive the success ratio for each plant individual. The *logit* link function was used in the model with 'treatment' (local/transferred) and 'altitude' (lowland/mountain) as the explanatory categorical variables. The computer software R (version 2·13·0, http://www.r-project.org/) was used for this analysis due to the suitability of the statistical package *Epi* (version 1·1·44, http://cran.r-project.org/package=Epi) for this dataset.

The following analyses were performed using IBM SPSS Statistics 20·0 (IBM SPSS, 2011). To test whether there were significant differences in morphology trait values and floral scent compounds between the populations and altitudes, and in which traits and compounds, generalized linear models (GLMs) were conducted separately for each morphological trait and scent compound, using 'population' nested within 'altitude' and 'altitude' as factors.

For floral colour comparison a principal component analysis (PCA) was performed to reduce the large number of values into a few orthogonal variables (principal components, PCs). The PCA was conducted with standardized values, using varimax rotation and extracting components with eigenvalues greater than 1. The PC scores were entered into two-way ANOVAs, examining whether there was significant difference in colour wavelength composition of individuals between populations and altitudes.

A four-way ANOVA was used to evaluate whether the variation in nectar sugar concentration was due to the altitudinal difference, or the abiotic factors humidity and temperature. In the model, sugar concentration was the response variable with the factors 'altitude', 'temperature' and 'humidity' as the explanatory variables.

RESULTS

Pollinator guilds in the lowland and mountains

The identification of all caught and observed pollinators (196 individuals in total) is reported only to the genus level, due to uncertain species-level identification of individuals of some genera (*Polyommatus*, *Adscita*, *Zygaena* and *Stenoptilia*). Likewise, one case was reduced to family level (Pterophoridae) and two cases to order level (Diptera and Coleoptera).

In the lowland populations, pollinators of two insect orders Lepidoptera and Coleoptera were observed (Fig. 2A). In the order Lepidoptera, a total of four butterfly families and four moth families were found, with the most frequent pollinators being the butterfly species *Ochlodes sylvanus* (Esp.) at 26·63 % of all pollinator visitations per hour and the moth *Phytometra viridaria* (Cl.) at 24·43 % of all pollinators h^{-1}. Neither of these pollinators visited plants in the mountains.

The pollinators observed in the mountain populations belonged to the three insect orders Lepidoptera, Diptera and Coleoptera (Fig. 2B). The order Lepidoptera was the most abundant, characterized by pollinators belonging to eight moth and three butterfly families. The most common pollinator was *Eudonia sudetica* (Z.), which comprised 28·65 % of all pollinators h^{-1} and was not observed to visit lowland plants. The functional group Diptera, which made up 8·47 % of all pollinators h^{-1} in the mountains, was also absent in the lowlands. Identification revealed that eight out of 11 Diptera specimens were species of the family Empididae.

The overlap of pollinator guilds between the lowland and mountain populations was minor, including individuals of the genus *Polyommatus* and the family Pterophoridae. The pollinators of the genus *Polyommatus* were one of the major pollinator groups of mountain flowers but made up only 6·14 % of the lowland pollinators h^{-1}. Pollinators of the family Pterophoridae made up 16·27 % of the mountain pollinators h^{-1}, but only 2·05 % of the lowland pollinators h^{-1}. In addition, species of the genus *Pieris* and the order Coleoptera were observed in both the lowlands and the mountains, but the visitation rate was very low at only one observation in each altitudinal region.

Nocturnal pollinators visited at a rate of 1·95 pollinators h^{-1} in the mountains, but at only 0·27 pollinators h^{-1} in the lowlands. The frequency of total pollinator observations in the mountains (1·96 pollinators h^{-1}) was approximately four times higher than that of the lowlands (0·46 pollinators h^{-1}).

Transfer experiment

For the vertical transfers, the reproductive success of lowland individuals was not significantly different whether they stayed in the lowlands or were transferred to the mountains ($z_{106} = 1·04$, $P = 0·299$; Fig. 3A), while the reproductive success of mountain individuals was significantly lowered when moved to the

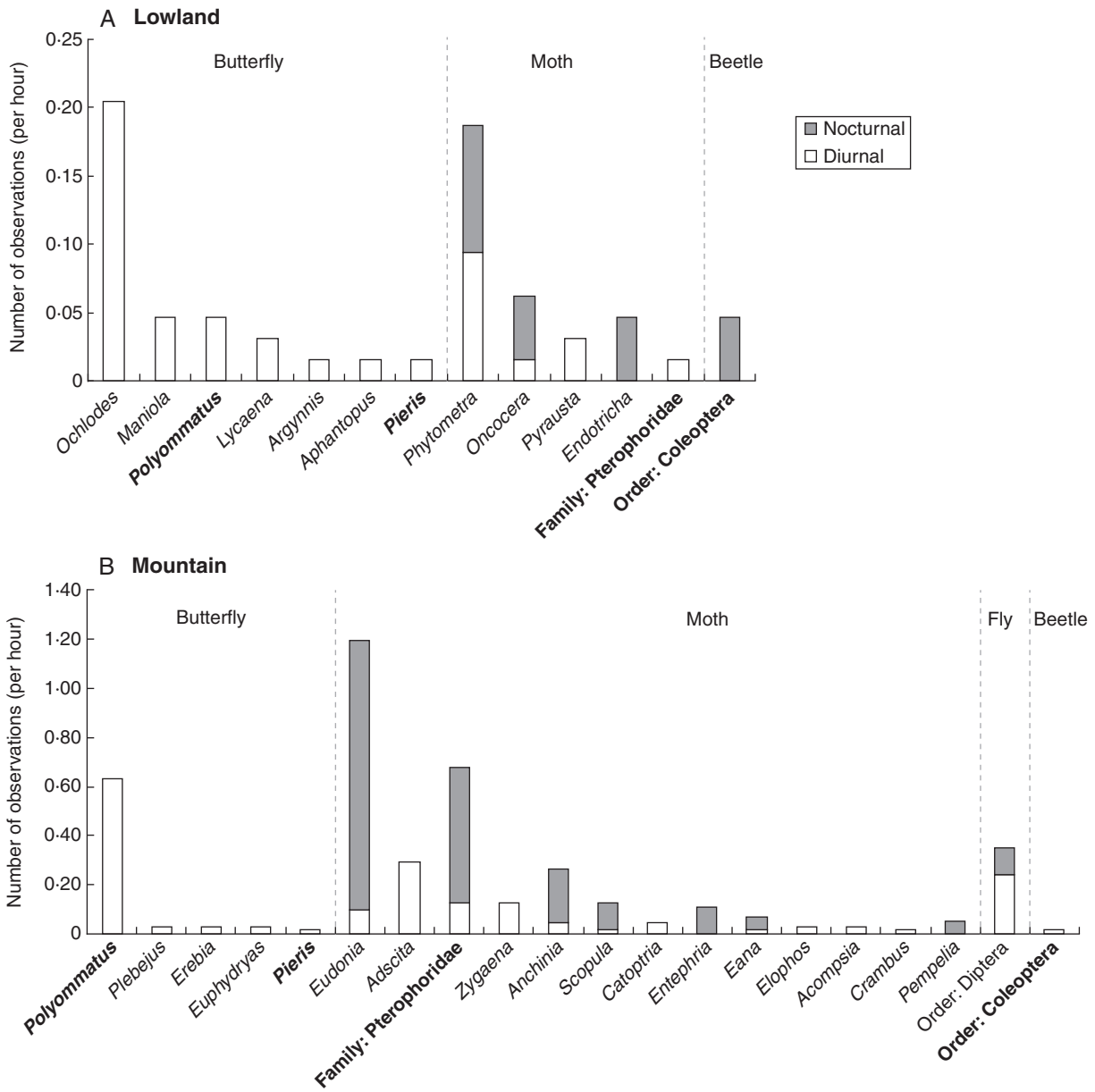

FIG. 2. The rate of pollinator individuals visiting *G. odoratissima* flowers measured as the number of pollinator observations per hour for (A) the lowland populations and (B) the mountain populations for each pollinator genus unless otherwise stated. The overlapping pollinators between the altitudinal regions are marked in bold.

lowlands compared with the left behind 'local' mountain individuals ($z_{107} = -4{\cdot}05$, $P < 0{\cdot}001$). Furthermore, there was a statistically significant difference between reproductive success of 'local' and 'transferred' individuals in both altitudinal regions. Mountain plants had consistently higher reproductive success than lowland plants. On the one hand, the mean female reproductive success for 'transferred' mountain individuals was significantly higher than that of the 'local' lowland individuals ($z_{127} = 5{\cdot}84$, $P < 0{\cdot}001$). On the other hand, the difference in mean reproductive success was also significantly greater for the 'local' mountain individuals, which received over three times higher reproductive success compared with the 'transferred' lowland individuals ($z_{86} = -8{\cdot}04$, $P < 0{\cdot}001$).

For the horizontal transfers, there was no significant difference in the reproductive success of lowland individuals between their 'local' populations and their 'transferred' populations ($z_{85} = 1{\cdot}90$, $P = 0{\cdot}057$; Fig. 3B), nor was there for mountain individuals ($z_{81} = 0{\cdot}80$, $P = 0{\cdot}421$). Moreover, there was no significant difference between the 'local' or 'transferred' individuals within the lowland populations ($z_{63} = 0{\cdot}95$, $P = 0{\cdot}342$) and the mountain populations ($z_{103} = 1{\cdot}48$, $P = 0{\cdot}139$). No significant difference was found between the reproductive success of the 'local' lowland and 'local' mountain individuals ($z_{82} = -0{\cdot}27$, $P = 0{\cdot}786$). There was no effect of the factor 'plot' on reproductive success in all populations ($z_{209} = -0{\cdot}22$, $P = 0{\cdot}826$).

In the hand-pollinated plants, there was no significant difference between the number of hand-pollinated flowers and the number of subsequent fruit set in both the lowland ($t_{18} = -1{\cdot}46$, $P = 0{\cdot}163$) and the mountain populations ($t_2 = -2$, $P = 0{\cdot}184$).

Floral morphology differences between altitudes

With regard to altitude differences, the mean morphological trait measurements of lowland flowers were all greater than those of the mountain flowers (Table 1). Flower width, labellum width, side lobe length and flower area were significantly larger in the lowlands. However, when the traits were standardized to the length of the inflorescence, all traits were significantly larger in the mountain populations than in the lowland populations (Table 1). Additionally, there were differences in absolute (Table S3 and Fig. S1) and standardized trait means among some populations (Table S4).

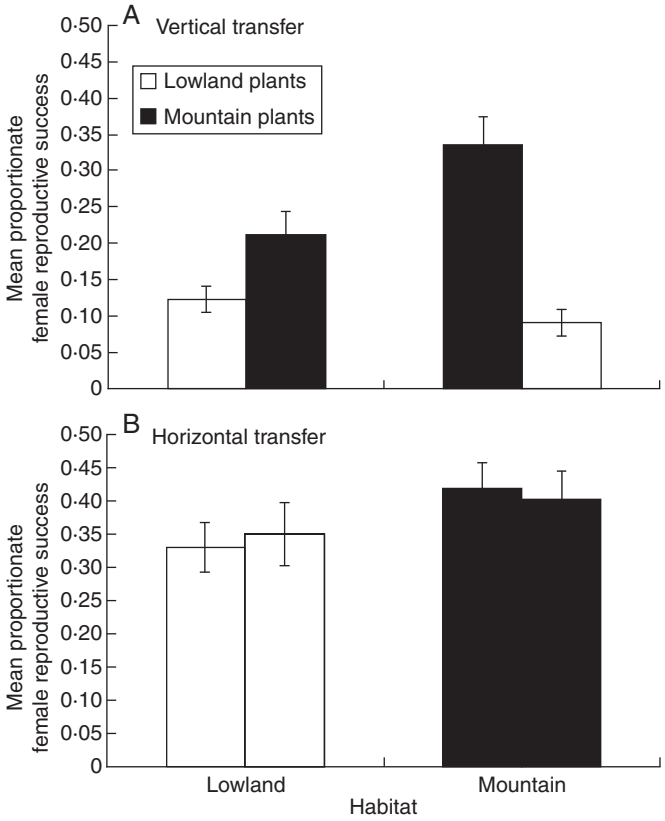

FIG. 3. The mean (± 1 s.e.) proportionate female reproductive success from (A) vertical transfers and (B) horizontal transfers in the lowland and mountain populations. For each pair of bars, left bar: 'local' individuals, right bar: 'transferred' individuals.

Floral scent differences between altitudes

The mean sum of the absolute amounts of compounds per inflorescence was higher in the lowlands (mean ± s.d. = 4392·75 ± 3776·79 ng L^{-1}) than in the mountains (3160·87 ± 2363·35 ng L^{-1}) ($t_{195·57} = 3·07$, $P = 0·002$). However, there was no difference in the mean amount of compounds emitted per flower between the lowland and the mountain populations ($t_{248·01} = -1·64$, $P = 0·1$).

There were significant differences in relative amounts for 18 of the 22 compounds in the scent emission between the altitudes (Fig. 4). There were significant population differences for all 22 compounds, apart from hexyl acetate and methyl eugenol (Table S6 and Fig. S2).When considering aromatic compounds and other compounds separately, the lowland populations had significantly higher relative amounts of nine out of 12 aromatic compounds compared with the mountain populations (Fig. 4A). By contrast, the mountain populations had significantly higher relative amounts of nine out of 10 non-aromatic compounds compared with the lowland populations (Fig. 4B).

Floral colour differences between altitudes

The PCA produced four PCs with eigenvalues greater than 1, explaining 99·79 % of the total variance in the data. PC1 (43·47 % of variance) had significant loadings of wavelengths between 488 and 636 nm, PC2 (22·44 %) of wavelengths between 400 and 487 nm, PC3 (17·08 %) of wavelengths between 637 and 700 nm, and PC4 (16·80 %) of wavelengths between 350 and 399 nm.

There was only a significant difference in the relative reflectance of wavelengths in PC1 between the lowlands and mountains

TABLE 1. *Mean (± s.d.) morphological trait values and standardized morphological trait values for flowers of the lowland and mountain populations*

		Mean trait value			Standardized mean trait value		
Trait	*n*	Lowland	Mountain	GLM altitude, z_1	Lowland	Mountain	GLM standardized altitude, z_1
(1) Flower width	231	9·13 ± 1·39	8·55 ± 1·12	29·54***	14·46 ± 3·60	18·16 ± 6·95	21·30***
(2) Flower height	231	7·73 ± 1·10	7·58 ± 0·91	2·47	12·18 ± 2·90	16·02 ± 6·03	31·25***
(3) Labellum width	229	3·37 ± 0·60	3·11 ± 0·54	22·49***	5·30 ± 1·28	6·52 ± 2·43	18·55***
(4) Labellum height	232	3·77 ± 0·56	3·67 ± 0·48	3·71	5·96 ± 1·47	7·73 ± 2·83	29·78***
(6) Side-lobe length	228	2·70 ± 0·49	2·56 ± 0·44	8·69**	4·26 ± 1·00	5·38 ± 2·05	23·25***
(7) Interlobe distance	226	1·69 ± 0·23	1·66 ± 0·26	1·49	2·72 ± 0·77	3·41 ± 1·10	27·66***
(8) Spur length	226	4·55 ± 0·52	4·54 ± 0·55	0·00	7·29 ± 2·05	9·58 ± 3·47	35·28***
(9) Flower area	227	31·36 ± 8·81	29·45 ± 6·98	7·14**	48·17 ± 13·66	61·72 ± 24·65	22·36***
(10) Lobe length	228	1·16 ± 0·27	1·18 ± 0·24	0·15	1·85 ± 0·60	2·45 ± 0·94	29·36***

All units of absolute trait values are in mm, apart from 'flower area' (trait 9) which is measured in mm^2. Data for 'spur entrance to height of interlobe' (trait 5) were removed prior to the analysis as it was a negligible trait used to derive trait 10 'lobe length'. Results from the generalized linear models of the mean absolute and standardized trait values are shown for trait comparisons between the altitudinal regions. Traits that are significantly different are shown as $*P < 0·05$, $**P < 0·001$, $***P < 0·0001$.

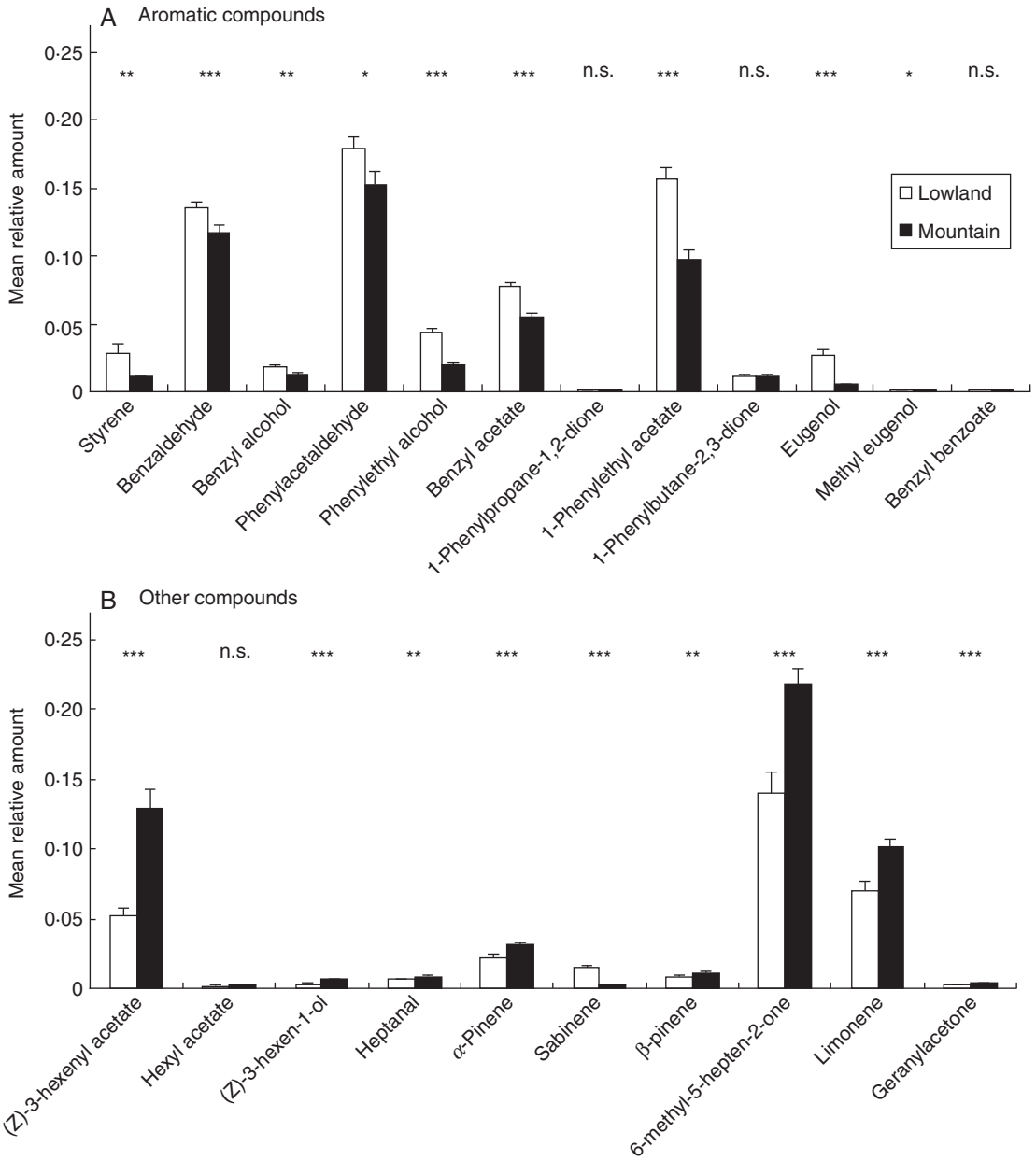

FIG. 4. The mean (± 1 s.e.) relative amount of volatile scent compounds separated into (A) aromatic compounds and (B) other compounds, for individuals in the lowland and mountain populations ($n = 254$ for all compounds). The standard IUPAC chemical nomenclature of these compounds can be found in Table S5. Comparisons of the relative quantity of each compound were made between the two altitudinal regions using generalized linear models. Compounds with significantly different relative amounts between the altitudes are shown as $*P < 0{\cdot}05$, $**P < 0{\cdot}001$, $***P < 0{\cdot}0001$ above the bars.

($F_{1,6} = 33{\cdot}07$, $P = 0{\cdot}001$), as well as among some populations ($F_{7,156} = 32{\cdot}68$, $P < 0{\cdot}001$; Fig. S3). Field observations showed lowland flower colours to range from deep pink to pink, and mountain flowers as being comparably lighter with colours from light pink to white (M. Sun, pers. obs.).

Nectar sugar concentration difference between altitudes

The mean sugar percentage in the lowland populations 'Döttingen' (mean $\pm$ s.d. $= 19{\cdot}75 \pm 3{\cdot}04$ %) and 'Linn' ($15{\cdot}95 \pm 3{\cdot}03$ %) were slightly higher than that of the mountain populations 'Münstertal' ($15{\cdot}59 \pm 2{\cdot}22$ %) and 'Schatzalp' ($11{\cdot}28 \pm 1{\cdot}91$ %). A significant influence of altitude ($F_{1,158} = 15{\cdot}47$, $P < 0{\cdot}001$) and population ($F_{2,158} = 7{\cdot}98$, $P < 0{\cdot}001$) on the sugar percentage of the nectar was indicated. There was also a significant influence of temperature at the time of measurement on the nectar sugar percentage ($F_{1,158} = 15{\cdot}62$, $P = 0{\cdot}014$), although there was no evidence of any effect of humidity ($F_{1,158} = 0{\cdot}49$, $P = 0{\cdot}642$). Thus, we conclude that although nectar sugar concentration differed between the altitudinal regions, temperature is likely to be responsible for this difference as opposed to pollinators.

DISCUSSION

Although pollinator adaptation is often believed to be a driving force for the evolution of floral trait variation, few studies have explicitly tested this assumption by reciprocal transfer experiments. In this study, we investigated pollinator adaptation in the orchid *G. odoratissima* over a broad altitudinal range by conducting transfer experiments. The results showed local pollinator adaptation in mountain plants, and as well as distinct differences in pollinator guilds, floral morphology, scent composition and colour between lowland and mountain populations. Horizontal transfers within lowland and mountain regions did not show any significant differences in pollination success, eliminating the possibility of population-specific adaptation.

Local pollinator adaptation

Our reciprocal transfer experiment strongly suggests that the observed differences in pollination success were caused by the different abilities of plants to attract pollinators in non-native regions, or by 'local' (native) pollinators depositing pollinia onto stigmas of 'transferred' (non-native) plants with lower efficiency (not measured here). Although the method of using cut plants for this experiment may alter the plant floral scent (Schiestl *et al.*, 1997), such physiological changes would have occurred in both the 'local' and the 'transferred' plants. As both these plant groups were subjected to the same treatment, there should not have been any systematic bias in the experiment.

We suggest that our transfer experiment results can be explained by considering the lowland and mountain plants as specific pollination ecotypes. Evidence for these altitude-specific ecotypes can be derived from observing the pollination success of plants moved to a non-native region. We found that the reproductive success of the lowland ecotype was not significantly different when they were moved to the mountain environment compared with their reproductive success in their 'local' lowland environment. Conversely, when the mountain ecotype was moved to the lowlands, it suffered a loss in reproductive success. As the reproductive success of the natural lowland plants and natural mountain plants are not significantly different, it cannot be said that the reduction in the reproductive success of the 'transferred' mountain plants was due to the lower pollinator visitation frequency in the lowlands. Rather, it could be explained in terms of the adaptation of mountain ecotypes to a relatively abundant functional group of pollinators that was not observed in the lowlands, namely the empidid flies. The absence of pollinating empidids may explain the decrease in reproductive success of mountain plants in the lowlands. Pollination by flies has been established before to be more abundant at higher altitudes (Arroyo *et al.*, 1982) and of greater importance in mountain plants compared with lowland plants (Müller, 1881; Dressler, 1993; Mani and Giddings, 1980), as they are thought to increase in importance in cooler climatic conditions (Faegri and van der Pijl, 1979; Warren *et al.*, 1988). Empidid flies are primarily predatory but their mouthpart is thought also to be well suited for extracting nectar from flowers with medium spur or corolla lengths. Empidid flies have previously been recorded as orchid pollinators, in particular as frequent diurnal visitors of *G. conopsea*, the sister species of *G. odoratissima*, in Norway (Sletvold *et al.*, 2012). The orchid *Platanthera stricta*, occurring in subalpine forests, is also thought to be pollinated by empidids (Patt *et al.*, 1989).

Pollinator guilds and floral trait differences

We observed significant differences between the lowland and mountain populations in the majority of the floral traits measured, together with pronounced differences in the pollinator communities. Aside from pollinator interactions, other biotic factors can influence floral and plant trait evolution, such as herbivores (Gómez and Zamora, 2000; Gómez, 2003; Strauss *et al.*, 2004), seed predators (Cariveau *et al.*, 2004; Carlson and Holsinger, 2010), nectar robbers (Galen and Cuba, 2001; Irwin *et al.*, 2001; Galen and Butchart, 2003) and competitors (Levin and Brack, 1995). Additionally, there are abiotic factors such as the environment and climate which may impact floral traits through, for example, drought (Galen, 2000) and heat stress (Coberly and Rausher, 2003). Our reciprocal transfer experiments, however, clearly document floral adaptations due to the different ability of plants to achieve pollination success through pollinator attraction. Herbivores or abiotic factors were not observed to cause any detrimental effects on the experimental plants. It is, however, unlikely that all measured traits contribute equally to pollinator adaptation, and thus the observed differences may represent an adaptive compromise to selection by pollinating and non-pollinating (biotic and abiotic) agents over a geographical area (as reviewed by Gómez and Zamora, 2000; Strauss and Whittall, 2006; Cosacov *et al.*, 2014).

One of the most pronounced discrepancies between the two altitudinal regions was the existence and quantity of empidid flies as pollinators in the mountains. These flies may impose different selection as compared with lepidopterans, due to their considerably different morphology and possible disparities in preference for floral signals. Floral scent was thought to be the primary attractant of empidid flies in *Plathanthera stricta*, where bioassays have shown that without a visual stimulus of the flower, the floral scent will elicit probing behaviour in these insects (Patt *et al.*, 1989). The scent compounds that *P. stricta* has in common with *G. odoratissima* are α-pinene, benzaldehyde, β-pinene, limonene, benzyl alcohol and phenylethyl alcohol (Patt *et al.*, 1988). Our results showed that half of these compounds (α-pinene, β-pinene and limonene) were more abundant in the mountain populations compared with the lowlands. However, further investigations are needed to understand more about potential selection by empidids on specific floral scent compounds, as well as on floral colour and morphology in the mountain *G. odoratissima.*

Besides the prevalence of empidids, we also noted the existence of more moth compared with butterfly pollinators in the mountain populations, in addition to qualitatively and quantitatively more nocturnal pollination. Most of these nocturnal pollinators belonged to the families Geometridae and Pyralidae, which are species operating predominantly at dawn or dusk and at night during warm summer weather (Willmer, 2011). Moth pollination is generally associated with plants with paler shades of floral colour, compared with the broader colour ranges of butterfly-pollinated flowers. Many studies have found that moth-pollinated species visited flowers that are white, cream or yellow (e.g. Oliveira *et al.*, 2004). Our observations are consistent with these results as flowers in the mountains were considerably lighter, in

contrast to lowland flowers. These light-coloured flowers could permit nocturnal pollinators to visually discern them more easily under very low light conditions. It has been documented that moth preferences switch from pink and yellow flowers in the early evening to exclusively white flowers in the night (Schremmer, 1941). However, establishing the location of flowers often required the aid of strong, sweet scent (Klahre *et al.*, 2011). We showed here that alpine plants emit relatively more non-aromatic compounds, while lowland populations emit greater relative amounts of most aromatic compounds. These differences could be due to dissimilar preferences of the pollinator communities. Soil nutrients may also play an important role, as most aromatic compounds analysed here are synthesized from phenylalanine as a start substrate (Dudareva *et al.*, 2013). While nitrogen is required for amino acid synthesis in plants, it is generally known that alpine plant productivity is constrained by the limited supply of nitrogen in mountain soil compared with the lowlands (Lütz, 2012). Thus, nitrogen limitation may explain some of the altitudinal differences in floral scent bouquets.

Apart from colour and scent, floral morphology is also a key trait for pollinator adaptation. Although flower dimensions were found to be larger in lowland plants, standardized trait values indicated that mountain flowers were significantly larger relative to their inflorescence size for all traits compared with the lowland flowers. This shows that alpine populations may allocate relatively more resources to display size, perhaps to compensate for the shorter flowering period in the mountains.

Implications of the study

The differences in plant traits between the altitudes are consistent with the hypothesis initially proposed by Grant and Grant (1965) and Stebbins (1970) that divergence in floral form is attributed to the variation in geographical pollinator mosaics. Our results agree with previous reports supporting this theory, such as a study by Miller (1981) which suggested that differentiation of flower colour and spur length in three geographically separated populations of *Aquilegia caerulea* is caused by differences in composition and abundance of hawkmoth species. Floral variation over different islands was shown by Martén-Rodríguez *et al.* (2011), where divergence in *Heliconia bihai* between two islands corresponded to differences in pollinators on the islands. Our study takes a further step from correlating floral traits with pollinator differences by confirming that trait differentiation are pollinator-driven through transfer experiments.

To better understand which traits underlie pollinator adaptations in plants, future studies should explore patterns of phenotypic selection on floral traits in different populations and regions. Furthermore, the molecular basis of adaptive traits, as well as the variability of adaptive genes in natural populations, needs to be investigated to improve our understanding of how patterns of variability allow adaptations to fluctuating pollinator environments. Such organismal and molecular micro-evolutionary studies may present vital contributions to understanding the processes of plant evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1. Geographical locations of the lowland and mountain populations of *G. odoratissima* within Switzerland, the year of pollinator observations and transfer experiments, and the year and sample size for floral phenotype measurements in each population. Table S2. Mean proportionate female reproductive success in each of the lowland and mountain populations used in the vertical and horizontal transfer experiments. Table S3. Mean of morphology trait values for all populations of the lowland and mountains. Fig. S1. Generalized linear model comparisons with Bonferroni post-hoc tests between all pairs of populations for each of the nine morphological traits. Fig. S2. Generalized linear model comparisons with Bonferroni post-hoc tests between all population pairs for each of the 22 scent compounds. Fig. S3. ANOVA comparisons with Bonferroni post-hoc test between all population pairs for PC1 from the floral colour PCA.

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Supplementary Data

Table S1. Geographic locations of the lowland and mountain populations of *G. odoratissima* within Switzerland, the year of pollinator observations and transfer experiments, and the year and sample size for floral phenotype measurements in each population.

Population	Geographic coordinates	Pollinator observation	Flower phenotype				Transfer experiment	
			Morphology	Scent	Colour	Nectar	Vertical transfer	Horizontal transfer
Lowland								
Döttingen	N: 47°34'28" E: 8°16'21" 500 m.a.s.l.	2010, 2012	2010 (n = 30)	2010 (n = 30)	2012 (n = 30)	2012 (n = 22)	2010	2011, 2012
Linn	N: 47°28'37" E: 8°7'1" 510 m.a.s.l.	2011	2010 (n = 30)	2010 (n = 30)	---	2012 (n = 20)	2012	2012
Nätteberg	N: 47°29'42" E: 8°05'47" 490 m.a.s.l.	2012	2012 (n = 26)	2012 (n = 30)	2012 (n = 30)	---	2010	2011
Remigen	N: 47°31'47" E: 8°09'42" 570 m.a.s.l.	2011, 2012						
Rossweid	N: 47°18'43" E: 8°30'42" 610 m.a.s.l.	2012	---	2011 (n = 30)	2012 (n = 16)	---	2012	---
Schnäggenwald	N: 47°24'26" E: 8°50'40" 640 m.a.s.l.	---	---	---	---	---	2012	---
Mountain								
Schatzalp	N: 46°48'21" E: 09°49'32" 1780 m.a.s.l.	2010, 2011, 2012	2010 (n = 30)	2010 (n = 30)	2012 (n = 6)	2012 (n = 20)	2010	2011
Preda	N: 46°35'24" E: 09°46'37" 1810 m.a.s.l.	2012	2012 (n = 29)	2012 (n = 21)	2012 (n = 21)	---	2010	2011
Albulapass	N: 46°34'54" E: 09°48'50" 2250 m a.s.l.	2010, 2012	2010 (n = 30)	2010 (n = 30)	2012 (n = 20)	---	2012	---
Cinuos-chel-Brail	N: 46°38'31" E: 10°01'37" 1610 m.a.s.l.	---	2012 (n = 27)	2012 (n = 23)	2012 (n = 21)	---	2012	2011
Münstertal	N: 46°47'48" E: 10°19'8" 1810 m.a.s.l.	2011, 2012	2010 (n = 30)	2010 (n = 30)	2012 (n = 20)	2012 (n = 20)	2012	2011
Corviglia	N: 46°30'22" E: 9°50'01" 2150 m a.s.l.	2011, 2012	---	---	---	---	---	---

Not all populations were available for pollinator observations, sampling of floral morphology, scent, colour, and transfer experiments due to (1) yearly variation in plant availability, (2) permit availability for each population, and (3) timing and logistic constraints.

Table S2. Mean (± SD) proportionate female reproductive success in each of the lowland populations Döttingen: 'D', Linn: 'L', Nätteberg: 'N', Rossweid: 'R', Schnäggenwald: 'SW', and mountain populations Albulapass: 'A', Cinuos-chel-Brail: 'C', Münstertal: 'M', Preda: 'P', Schatzalp: 'S', used in the vertical and horizontal transfer experiments. The pairs of populations used ('transferred' and 'local') and number of individuals per population included in the statistical analysis is also shown. '*T*' = transferred plants, '*L*' = local plants.

	Lowland										Mountain							
Vertical																		
Local population	L		D		N		SW		R		S		P		C		M	
Transferred population	A		S		P		M		C		D		N		R		SW	
Treatment	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*
n	16	16	13	14	10	15	15	15	16	16	13	12	15	13	15	15	15	15
Proportionate female reproductive success ± SD	0.23 ± 0.28	0.09 ± 0.10	0.09 ± 0.08	0.17 ± 0.20	0.23 ± 0.23	0.14 ± 0.13	0.22 ± 0.26	0.10 ± 0.18	0.31 ± 0.30	0.12 ± 0.12	0.17 ± 0.11	0.40 ± 0.19	0.08 ± 0.11	0.29 ± 0.29	0.00 ± 0.00	0.18 ± 0.13	0.06 ± 0.15	0.45 ± 0.32
Horizontal																		
Local population	L		D		N						S		P		C		M	
Transferred population	D		L		D						P		S		M		C	
Treatment	*T*	*L*	*T*	*L*	*T*	*L*					*T*	*L*	*T*	*L*	*T*	*L*	*T*	*L*
n	14	14	12	13	9	11					13	12	15	12	11	10	9	11
Proportionate female reproductive success ± SD	0.21 ± 0.29	0.23 ± 0.24	0.24 ± 0.16	0.31 ± 0.21	0.62 ± 0.15	0.45 ± 0.15					0.23 ± 0.27	0.22 ± 0.21	0.37 ± 0.30	0.28 ± 0.16	0.56 ± 0.32	0.59 ± 0.17	0.52 ± 0.16	0.63 ± 0.25

Data from the population of Albulapass in the vertical transfer experiment, and from Döttingen in the horizontal transfer experiment were excluded as all individuals died due to adverse weather conditions.

Table S3. Mean (± SD) of morphology trait values for all populations of the lowland and mountains. All measurements are in mm except for flower area, which is in mm^2. Generalised linear model comparisons between the populations are shown for each morphology trait.

Traits	GLM	Lowland populations			Mountain populations				
	z_6	Döttingen	Linn	Nätteberg	Albulapass	Cinuos-chel-Brail	Münstertal	Preda	Schatzalp
(1) Flower width	244.68***	9.77 ± 0.90	7.57 ± 0.73	10.19 ± 0.63	8.99 ± 1.17	8.11 ± 0.71	7.47 ± 0.70	9.10 ± 0.95	9.07 ± 0.96
(2) Flower height	164.34***	8.35 ± 0.75	6.52 ± 0.68	8.35 ± 0.57	7.91 ± 0.89	7.42 ± 0.61	6.80 ± 0.83	7.95 ± 0.83	7.81 ± 0.84
(3) Labellum width	135.47***	3.67 ± 0.39	2.75 ± 0.38	3.74 ± 0.40	3.26 ± 0.57	3.08 ± 0.36	2.65 ± 0.40	3.13 ± 0.44	3.38 ± 0.60
(4) Labellum height	109.82***	4.08 ± 0.40	3.23 ± 0.45	4.03 ± 0.34	3.7 ± 0.45	3.64 ± 0.39	3.30 ± 0.49	3.83 ± 0.44	3.87 ± 0.44
(6) Side-lobe length	84.94***	3.02 ± 0.33	2.26 ± 0.42	2.85 ± 0.32	2.72 ± 0.50	2.47 ± 0.36	2.31 ± 0.40	2.52 ± 0.39	2.73 ± 0.42
(7) Interlobe distance	79.75***	1.71 ± 0.21	1.52 ± 0.18	1.85 ± 0.17	1.66 ± 0.25	1.73 ± 0.22	1.43 ± 0.16	1.81 ± 0.23	1.67 ± 0.28
(8) Spur length	20.97**	4.72 ± 0.52	4.41 ± 0.54	4.51 ± 0.45	4.79 ± 0.47	4.72 ± 0.51	4.45 ± 0.47	4.44 ± 0.58	4.39 ± 0.63
(9) Flower area	249.65***	36.07 ± 5.12	20.94 ± 4.39	37.14 ± 4.84	32.3 ± 7.63	28.12 ± 4.06	21.95 ± 3.90	32.54 ± 5.31	32.27 ± 6.48
(10) Lobe length	58.68***	1.21 ± 0.31	1.01 ± 0.19	1.27 ± 0.21	1.06 ± 0.20	1.19 ± 0.274	1.05 ± 0.20	1.36 ± 0.23	1.22 ± 0.15

Traits that were significantly different are shown as *: $p<0.05$, **: $p<0.001$, ***: $p<0.0001$

Table S4. Mean (± SD) of standardised morphology trait values for all populations of the lowland and mountains. Generalised linear model comparisons between the populations are shown for each standardised morphology trait.

Standardised traits	GLM	Lowland populations			Mountain populations				
	z_6	Döttingen	Linn	Nätteberg	Albulapass	Cinuos-chel-Brail	Münstertal	Preda	Schatzalp
(1) Flower width	36.27***	12.12 ± 2.47	15.16 ± 4.08	16.35 ± 2.61	19.96 ± 7.58	13.15 ± 3.25	19.72 ± 8.46	18.46 ± 6.76	18.86 ± 5.77
(2) Flower height	33.03***	10.33 ± 2	13 ± 3.3	13.4 ± 2.22	17.52 ± 6.29	12.03 ± 3.08	17.99 ± 7.98	15.86 ± 5.69	16.15 ± 4.55
(3) Labellum width	30.15***	4.55 ± 0.94	5.46 ± 1.35	6 ± 1.1	7.18 ± 2.24	5.02 ± 1.46	7.08 ± 3.42	6.29 ± 2.42	6.88 ± 1.81
(4) Labellum height	30.30***	5.05 ± 1	6.44 ± 1.72	6.46 ± 1.12	8.18 ± 2.9	5.92 ± 1.61	8.66 ± 3.64	7.66 ± 2.79	7.99 ± 2.25
(6) Side-lobe length	31.40***	3.76 ± 0.7	4.48 ± 1.24	4.56 ± 0.78	5.98 ± 1.98	4.04 ± 1.24	6.02 ± 2.75	5.1 ± 1.94	5.61 ± 1.61
(7) Interlobe distance	32.00***	2.13 ± 0.41	3.06 ± 0.88	2.97 ± 0.56	3.62 ± 1.13	2.8 ± 0.79	3.53 ± 1.34	3.62 ± 1.17	3.41 ± 0.93
(8) Spur length	50.86***	5.82 ± 1.08	8.8 ± 2.25	7.23 ± 1.23	10.65 ± 3.05	7.7 ± 2.13	11.4 ± 4.34	8.85 ± 3.09	9.05 ± 3.25
(9) Flower area	40.29***	44.57 ± 9.54	41.55 ± 13.65	59.45 ± 10.79	70.78 ± 27.12	45.35 ± 12.01	58.84 ± 29.71	65.8 ± 25.02	65.94 ± 19.32
(10) Lobe length	27.60***	1.49 ± 0.41	2.04 ± 0.67	2.04 ± 0.51	2.38 ± 1.14	1.91 ± 0.59	2.63 ± 0.99	2.74 ± 0.95	2.53 ± 0.81

Traits that were significantly different are shown as *: $p<0.05$, **: $p<0.001$, ***: $p<0.0001$

Table S5. The chemical compound names use in this study and their chemical nomenclature under the International Union of Pure and Applied Chemistry (IUPAC).

Compound name	**IUPAC nomenclature**
(*Z*)-3-Hexen-1-ol	(*Z*)-Hex-3-en-1-ol
Styrene	Phenylethene
Heptanal	Heptanal
α-Pinene	(1*S*,5*S*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene((−)-α-Pinene)
Benzaldehyde	Benzaldehyde
Sabinene	4-Methylene-1-(1-methylethyl)bicyclo[3.1.0]hexane
β-Pinene	6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane
6-Methyl-5-hepten-2-one	6-Methylhept-5-en-2-one
(Z)-3-Hexenyl acetate	(Z)-3-Hexenyl acetate
Hexyl acetate	Hexyl acetate
Limonene	1-Methyl-4-(1-methylethenyl)-cyclohexene
Benzyl alcohol	Phenylmethanol
Phenylacetaldehyde	2-Phenylacetaldehyde
Phenylethyl alcohol	2-Phenylethanol
Benzyl acetate	Benzyl acetate
1-Phenylpropane-1,2-dione	1-Phenylpropane-1,2-dione
Phenylethyl acetate	1-Phenylethyl acetate
1-Phenylbutane-2,3-dione	1-Phenylbutane-2,3-dione
Eugenol	4-Allyl-2-methoxyphenol
Methyl eugenol	1,2-Dimethoxy-4-prop-2-en-1-ylbenzene
Geranyl acetone	(5*E*)-6,10-Dimethylundeca-5,9-dien-2-one
Benzyl benzoate	Benzyl benzoate

Table S6. Mean (± SD) relative amounts of volatile scent compounds of an inflorescence for all populations of the lowland and mountains. Generalised linear model comparisons between the populations are shown for each compound.

Compound	GLM	Lowland				Mountain				
	z_7	Döttingen	Linn	Nätteberg	Rossweid	Albulapass	Cinuos-chel-Brail	Münstertal	Preda	Schatzalp
(*Z*)-3-Hexen-1-ol	59.04***	0.40 ± 0.52	0.58 ± 0.76	0.16 ± 0.19	0.13 ± 0.43	1.32 ± 1.22	0.27 ± 0.31	0.70 ± 0.58	0.42 ± 0.31	0.41 ± 0.67
Styrene	58.75***	0.23 ± 0.17	0.71 ± 0.38	7.81 ± 12.07	2.79 ± 4.41	0.86 ± 0.43	0.83 ± 1.06	2.15 ± 0.91	1.18 ± 1.03	0.49 ± 0.56
Heptanal	192.12***	0.48 ± 0.38	0.84 ± 0.23	0.17 ± 0.14	0.95 ± 0.95	0.91 ± 0.35	0.24 ± 0.23	1.84 ± 1.01	0.40 ± 0.30	0.66 ± 0.36
α-Pinene	108.08***	1.16 ± 0.62	3.55 ± 3.28	3.27 ± 3.00	0.83 ± 0.57	1.72 ± 0.77	3.55 ± 2.41	5.52 ± 2.61	2.59 ± 1.65	2.15 ± 1.46
Benzaldehyde	190.90***	16.25 ± 4.32	9.50 ± 4.12	12.46 ± 4.93	15.78 ± 4.69	16.46 ± 6.08	4.89 ± 3.15	10.18 ± 4.02	7.38 ± 3.70	16.74 ± 5.00
Sabinene	291.39***	4.37 ± 3.00	0.76 ± 0.74	0.26 ± 0.25	0.46 ± 0.80	0.21 ± 0.11	0.21 ± 0.17	0.38 ± 0.20	0.17 ± 0.11	0.19 ± 0.14
β-Pinene	93.58***	0.82 ± 0.47	1.62 ± 1.72	0.80 ± 0.71	0.14 ± 0.15	0.51 ± 0.24	1.97 ± 1.26	1.23 ± 0.59	1.37 ± 1.00	0.75 ± 0.43
6-Methyl-5-hepten-2-one	612.86***	11.30 ± 6.31	39.50 ± 11.93	1.77 ± 1.16	3.24 ± 3.32	19.57 ± 10.56	15.48 ± 8.61	40.22 ± 10.86	13.01 ± 8.18	16.69 ± 8.03
(Z)-3-Hexenyl acetate	37.60***	7.19 ± 7.21	6.50 ± 7.07	5.03 ± 6.59	2.01 ± 1.99	21.07 ± 14.32	13.22 ± 19.77	5.06 ± 3.77	14.11 ± 14.56	11.66 ± 14.64
Hexyl acetate	14.07	0.25 ± 0.85	0.20 ± 0.13	0.05 ± 0.05	0.22 ± 0.30	0.31 ± 0.21	0.28 ± 0.21	0.31 ± 0.16	0.17 ± 0.14	0.13 ± 0.08
Limonene	120.94***	4.09 ± 1.52	11.03 ± 6.86	4.45 ± 3.79	8.45 ± 10.98	9.97 ± 5.06	9.25 ± 5.82	18.58 ± 7.15	5.99 ± 3.62	5.29 ± 3.17
Benzyl alcohol	59.70***	1.60 ± 1.12	1.03 ± 1.07	2.00 ± 1.26	2.81 ± 1.83	2.15 ± 1.89	0.53 ± 0.49	0.53 ± 0.61	1.40 ± 1.86	1.81 ± 1.59
Phenylacetaldehyde	146.97***	26.29 ± 7.97	12.13 ± 7.45	17.31 ± 7.34	16.00 ± 7.70	11.64 ± 4.91	20.38 ± 11.99	5.41 ± 3.81	17.95 ± 10.84	23.02 ± 9.00
Phenylethyl alcohol	109.43***	3.61 ± 1.99	1.75 ± 1.43	3.96 ± 2.33	8.13 ± 5.77	1.54 ± 1.52	2.87 ± 2.50	0.82 ± 0.82	3.14 ± 2.10	2.31 ± 2.07
Benzyl acetate	99.85***	7.09 ± 1.92	3.94 ± 2.21	9.11 ± 3.78	10.79 ± 5.66	6.17 ± 4.02	3.74 ± 2.50	2.48 ± 2.43	8.03 ± 6.91	7.40 ± 2.59
1-Phenylpropane-1,2-dione	40.23***	0.05 ± 0.02	0.04 ± 0.02	0.09 ± 0.06	0.08 ± 0.05	0.07 ± 0.03	0.05 ± 0.03	0.08 ± 0.04	0.09 ± 0.05	0.07 ± 0.03
Phenylethyl acetate	270.30***	13.20 ± 3.78	4.89 ± 2.94	21.66 ± 8.90	22.81 ± 9.92	4.87 ± 3.22	19.42 ± 10.71	3.76 ± 2.99	16.64 ± 8.38	8.49 ± 3.06
1-Phenylbutane-2,3-dione	273.58***	0.55 ± 0.37	0.18 ± 0.15	3.18 ± 2.13	0.83 ± 0.73	0.19 ± 0.12	1.64 ± 1.11	0.17 ± 0.20	3.46 ± 2.39	1.09 ± 0.89
Eugenol	81.60***	0.92 ± 0.66	0.78 ± 1.01	6.23 ± 7.65	3.05 ± 2.20	0.23 ± 0.22	0.38 ± 0.34	0.19 ± 0.18	1.84 ± 1.69	0.48 ± 0.54

Methyl eugenol	9.39	0.02 ± 0.05	0.08 ± 0.20	0.02 ± 0.02	0.11 ± 0.36	0.02 ± 0.06	0.00 ± 0.00	0.03 ± 0.09	0.00 ± 0.00	0.03 ± 0.09
Geranyl acetone	81.31***	0.11 ± 0.08	0.30 ± 0.14	0.20 ± 0.21	0.37 ± 0.63	0.18 ± 0.14	0.79 ± 0.75	0.31 ± 0.14	0.66 ± 0.42	0.13 ± 0.08
Benzyl benzoate	16.68*	0.02 ± 0.07	0.08 ± 0.22	0.01 ± 0.02	0.01 ± 0.06	0.01 ± 0.04	0.00 ± 0.00	0.05 ± 0.12	0.00 ± 0.00	0.03 ± 0.10

Significant differences are shown as *: $p<0.05$, **: $p<0.001$, ***: $p<0.0001$

Figure S1. Generalised linear model comparisons with Bonferroni post-hoc tests between all pairs of populations for each of the nine morphological traits. Results shown from population paired comparisons where light grey = not significant, dark grey = significant difference. The codes for the lowland populations are Döttingen: ‘D’, Linn: ‘L’, Nätteberg: ‘N’, and the codes for the mountain populations are Albulapass: ‘A’, Cinuos-chel-Brail: ‘C’, Münstertal: ‘M’, Preda: ‘P’, Schatzalp: ‘S’.

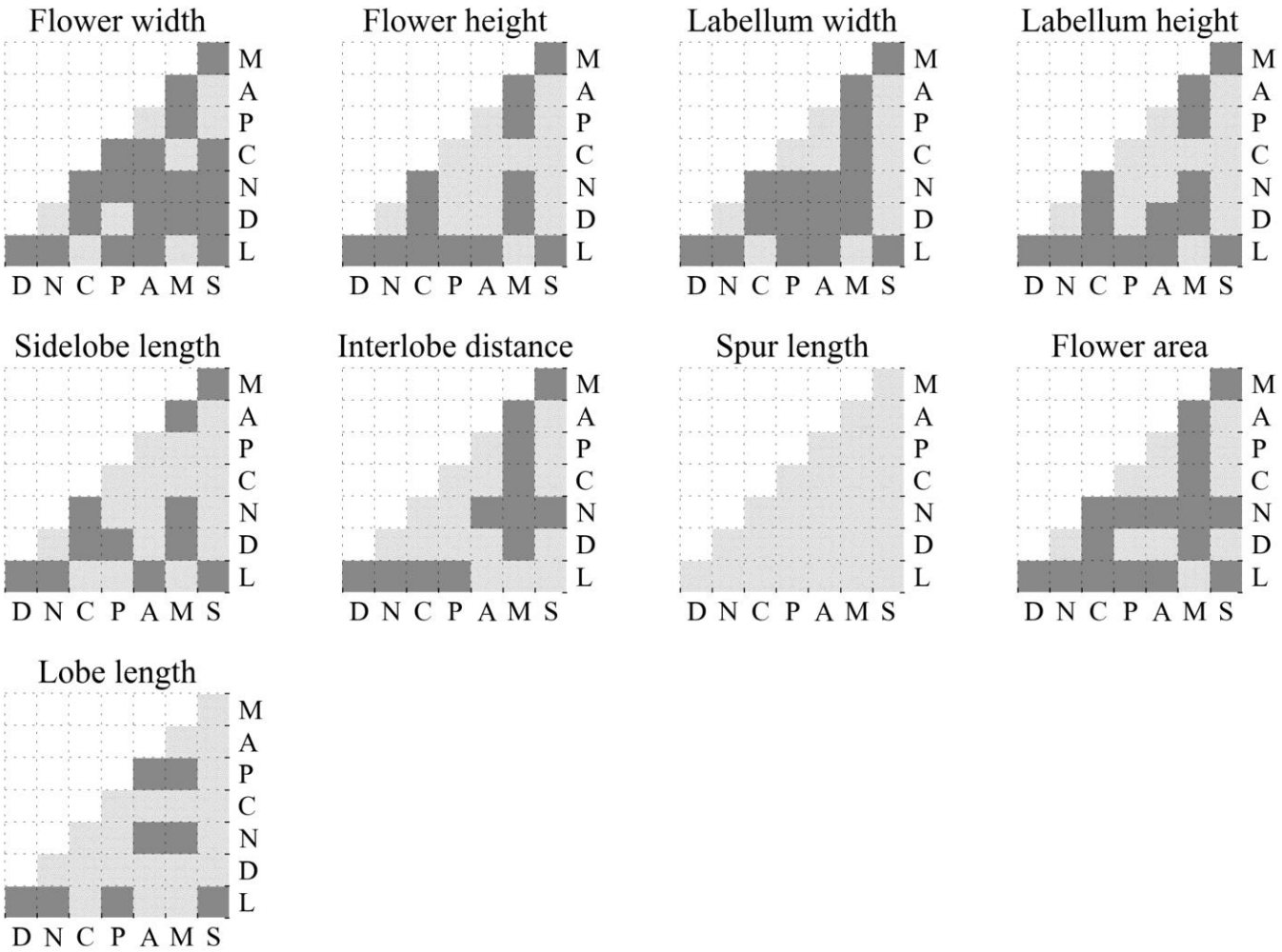


Figure S2. Generalised linear model comparisons with Bonferroni post-hoc tests between all population pairs for each of the 22 scent compounds (refer to Table S5 for official names). Results shown from population paired comparisons where light grey = not significant, black = significant difference. The population codes are Döttingen: 'D', Linn: 'L', Nätteberg: 'N', Rossweid: 'RW', Albulapass: 'A', Cinuos-chel-Brail: 'C', Münstertal: 'M', Preda: 'P', Schatzalp: 'S'.

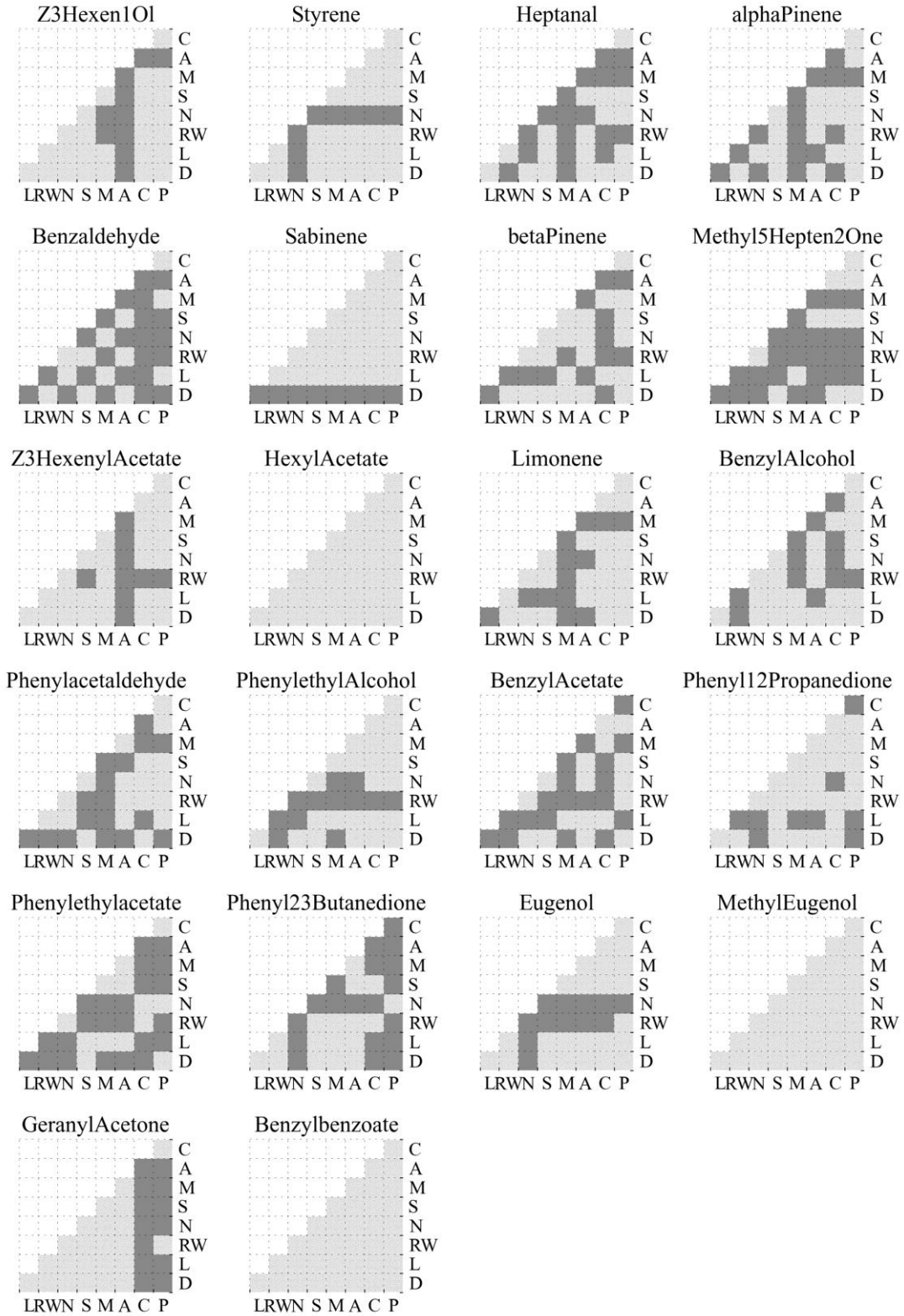
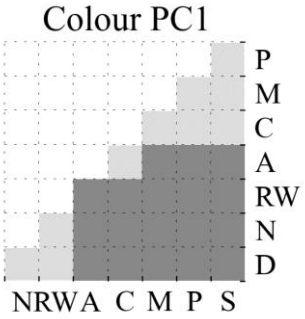


Figure S3. ANOVA comparisons with Bonferroni post-hoc test between all population pairs for PC1 from the floral colour PCA. Results shown from population paired comparisons where light grey = not significant, black = significant difference. The population codes are Döttingen: ‘D’, Linn: ‘L’, Nätteberg: 5 ‘N’, Rossweid: ‘RW’, Albulapass: ‘A’, Cinuos-chel-Brail: ‘C’, Münstertal: ‘M’, 6 Preda: ‘P’, Schatzalp: ‘S’.



Chapter 4.

FLORAL ISOLATION IS THE MAJOR REPRODUCTIVE BARRIER BETWEEN A PAIR OF REWARDING ORCHID SPECIES

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Author contributions
MS and FPS designed the experiment, PMS and KG also helped with the design
MS performed the majority of the field experiments, KG also helped with the experiments
MS performed the field experiment data analysis, KG performed the scent analysis
PMS performed the phylogenetic analysis
MS wrote the paper, significant contributions were made by FPS and PMS

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Floral isolation is the major reproductive barrier between a pair of rewarding orchid sister species

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Keywords:

floral scent;
pollination;
prepollination barriers;
reproductive isolation;
speciation.

Abstract

The crucial role of reproductive isolation in speciation has long been recognized; however, a limited number of studies quantify different isolation barriers and embed reproductive isolation in a phylogenetic context. In this study, we investigate reproductive isolation between the often sympatrically occurring orchid species, *Gymnadenia conopsea* and *G. odoratissima*. We examine the phylogenetic relationship between the two species and analyse floral isolation, fruit set and seed viability from interspecies crosses, as well as the ploidy level. Additionally, we quantify interspecies differences in floral signals and morphology. The results suggest that the two species have a sister–species relationship. In terms of reproductive isolation, we found complete floral isolation between the two species, but little to no post-pollination isolation; the species also mostly had the same ploidy level in the studied populations. We also show clear distinctions in floral signals, as well as in floral size and spur length. We propose that respective adaptation to short- vs. long-tongued pollinators was the driver of speciation in the here studied *Gymnadenia* species. Our study supports the key role of floral isolation in orchid speciation and shows that floral isolation is not restricted to highly specialized pollination systems, but can also occur between species with less specialized pollination.

Introduction

Speciation is one of the most fundamental processes in evolutionary biology and has remained both intriguing and, in some aspects, controversial (Coyne, 1994; Turelli *et al.*, 2001; Coyne & Orr, 2004). Speciation is commonly regarded as the evolution of reproductive isolation between previously interbreeding populations (i.e. the Biological Species Concept; Coyne *et al.*, 1988; Mayr, 1942). Reproductive isolation barriers can be classified according to their order of occurrence in reproduction. In plants, they include prepollination, prezygotic mechanisms such as phenological and floral isolation, and post-zygotic mechanisms such as hybrid (seed) inviability and hybrid sterility (Dobzhansky, 1937; Mayr, 1947; Grant, 1971). In ecological speciation, differential adaptation to environmental factors in incipient species leads to divergence and ultimately to reproductive isolation as a by-product (Schluter, 2001). Understanding the mechanisms of how adaptation leads to reproductive isolation has become a fundamental topic in speciation research (Rundle & Nosil, 2005). Determining the relative importance of different types of reproductive barriers acting between species can provide insights into the type of traits involved in adaptive divergence and the possible selective agents (Ramsey *et al.*, 2003; Coyne & Orr, 2004; Lowry *et al.*, 2008; Xu *et al.*, 2011).

Reproductive barriers are considered to act sequentially, with prezygotic barriers often acting as the critical initial filter against gene exchange, thus having a larger effect on total isolation (Widmer *et al.*, 2009). Within the prezygotic barriers in plants, floral isolation is regarded as a central one, exemplified in studies of *Mimulus* (Ramsey *et al.*, 2003), *Aquilegia* (Hodges & Arnold, 1994; Fulton & Hodges, 1999), *Petunia* (e.g. Dell'Olivo *et al.*, 2011) and *Ophrys* (Scopece *et al.*, 2007; Xu *et al.*, 2011). The importance of floral isolation

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Data archival location: submitted to GenBank.

supports the key role of floral traits and pollinators in speciation and/or coexistence of species in sympatry. The traits that contribute to floral isolation are varied and include floral colour from extensive studies in *Mimulus* (Schemske & Bradshaw, 1999) and *Aquilegia* (Hodges *et al.*, 2002), floral scent in a range of orchids (e.g. Schiestl & Ayasse, 2002; Xu *et al.*, 2011) and within other plant families (e.g. Waelti *et al.*, 2008; Hentrich *et al.*, 2010), and floral morphologies such as flower size (e.g. Schemske & Bradshaw, 1999), orientation and spur length (e.g. Fulton & Hodges, 1999). Although it is evident that prezygotic isolation is an important component in many study systems, the speed at which it evolves as compared to post-zygotic isolation is unclear and likely to be reliant on the genetic architecture or number of genes involved (Widmer *et al.*, 2009). Currently, known plant species with strong floral isolation are characterized by either a shift between major groups of pollinators (e.g. from bee to bird as in *Mimulus*, or from bee to moth pollination as in *Petunia* and *Aquilegia*) or highly specialized pollination (as in *Ophrys*). It is unclear, however, whether functional specialization to different pollinators within major taxonomic groups (e.g. long or short proboscis moths) can also lead to strong floral isolation.

In this study, we explored the mechanisms of reproductive isolation between *Gymnadenia conopsea* and *G. odoratissima*, which are two often sympatrically growing species of food-rewarding orchids. Orchids of the genus *Gymnadenia* mainly attract different groups of Lepidoptera species for pollination, with no apparent overlap in pollinators previously observed (Huber *et al.*, 2005; Vöth, 2000). In natural populations, few F1 hybrids can be found (Reinhard *et al.*, 1991), suggesting that reproductive isolation is strong. Our primary goal was to determine the strength of different isolation barriers and identify traits that may contribute to floral isolation. We put this study in a phylogenetic context to make inferences as to whether the mechanisms of reproductive isolation were relevant in the speciation process of the two *Gymnadenia* species. Thus, we address the following questions: (1) What is the phylogenetic relationship between the two species? (2) How strong is floral isolation in relation to post-pollination barriers? (3) Which floral traits differ between the species and, thus, potentially contribute to the reproductive isolation between them?

Materials and methods

Study system

Gymnadenia conopsea (L.) R.Br. s.l. (Orchidaceae) is distributed widely throughout Eurasia, whereas *G. odoratissima* (L.) Rich. is comparatively more sparsely distributed, found in temperate and mountainous regions of Europe. Both species are terrestrial orchids, inhabiting calcareous areas ranging from lowland forests to subalpine meadows of up to 2600 m a.s.l. These species produce nectar in floral spurs (Fig. 1). The pollination system of both species is considered functionally specialized, with the pollinators being mainly diurnal and nocturnal long- and short-tongued Lepidoptera species (Vöth, 2000; Sun *et al.*, 2014). *G. odoratissima* is pollinated by insects from over 29 genera and four functional groups (butterflies, moths, flies and beetles), whereas *G. conopsea* is visited by pollinators from approximately 42 genera and three functional groups (butterflies, moths and beetles) (Vöth, 2000; and references within). Previous phylogenetic analyses within the genus *Gymnadenia* suggested *G. conopsea* and *G. odoratissima* are sister species, albeit only one or very few samples of these species were included, none of them from our study area in Switzerland (Bateman *et al.*, 2003, 2006). We, therefore, performed an additional phylogenetic analysis with five nuclear markers to clarify the relationship between the two studied *Gymnadenia* species, using several accessions from our study populations (for details see supporting information). In this phylogenetic analysis, we also included *G. densiflora* (Wahlenb.) Dietrich (cf. Marhold *et al.*, 2005), which closely resembles *G. conopsea*, but differs from this species in flowering phenology, scent composition, habitat preferences and internal transcribed spacer (ITS) sequences (Gustafsson, 2000; Gustafsson & Lönn, 2003; Marhold *et al.*, 2005; Jersáková *et al.*, 2010; Stark *et al.*, 2011). In addition, we included *Gymnadenia* (= *Nigritella*) *rhellicani*, (Teppner & Klein, 1990) as well as the two *Dactylorhiza* species *D. majalis* and *D. maculata* as outgroups. The genus *Dactylorhiza* was chosen as an outgroup as it is known to be the sister genus

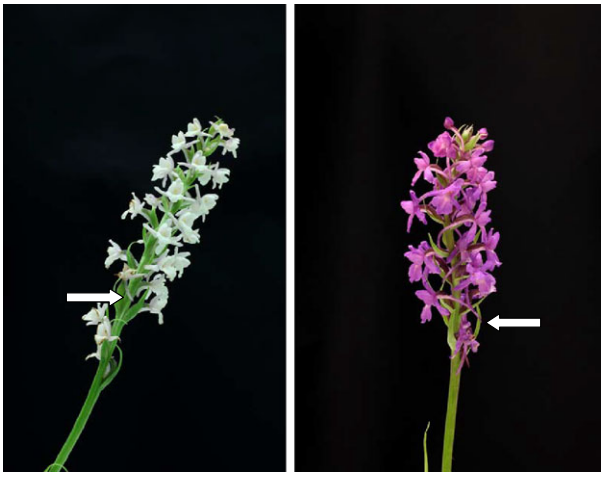

Fig. 1 An inflorescence of *Gymnadenia odoratissima* (left) and *G. conopsea* (right) from one of our study populations. The large difference in spur length (shown by an arrow) and size of the labellum are discernible.

of *Gymnadenia* (Bateman *et al.*, 2003, 2006). The study was conducted in a total of eleven natural populations in Switzerland (Fig. 2; Table S1). All *G. conopsea* and *G. odoratissima* populations were sympatric.

Phylogenetic analysis

Plant material from 24 individuals was collected in nine *Gymnadenia* populations in Switzerland during their flowering time in 2010. Leaf tissue samples were cut from each individual, placed in separate plastic bags and stored at −80 °C until analysis. DNA was extracted using the CTAB procedure following a slightly modified protocol of Doyle & Doyle (1990) and the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). In total, DNA was amplified from five nuclear markers, namely internal transcribed spacers 1 and 2 (ITS1, ITS2), anthocyanin O-methyltransferase (*AOMT*), chalcone synthase (*CHS*) and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*). *AOMT*, *CHS* and *G3PDH* markers contained both intron and exon sequence, putatively derived from single gene copies. Primers and amplification conditions are listed in Table S2. PCR products were cleaned prior to sequencing following the method of Werle *et al.* (1994). Sanger sequencing was carried out using BigDye 3.1 on an ABI PRISM 3130xl sequencing system (Applied Biosystems, Foster City, CA, USA) using the manufacturer's protocols. Rare ambiguous bases in sequencing data, such as single nucleotide polymorphisms between alleles within an individual, were encoded in IUPAC ambiguity notation. All sequences obtained in this study are available from Genbank under accession numbers KP225165 - KP225272.

Sequences were aligned using MUSCLE (Edgar, 2004) with manual adjustments in BioEdit 7.0.9.0 (Hall, 1999) and used for phylogenetic analysis by Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Appropriate nucleotide substitution models (Table S3) were inferred using MrModeltest 2.3 (Nylander *et al.*, 2004) and the Akaike information criterion. BI in MrBayes (two runs, four MCMC chains per run) was automatically stopped when the standard deviation of split frequencies fell below 0.01 (633 000 generations) and accepted when the potential scale reduction factor (PSRF) (Brooks & Gelman, 1998) for all parameters was 1 ± 0.01, indicating convergence of both runs. Trees were sampled every 1000 generations, with the first 100 samples discarded as burn-in.

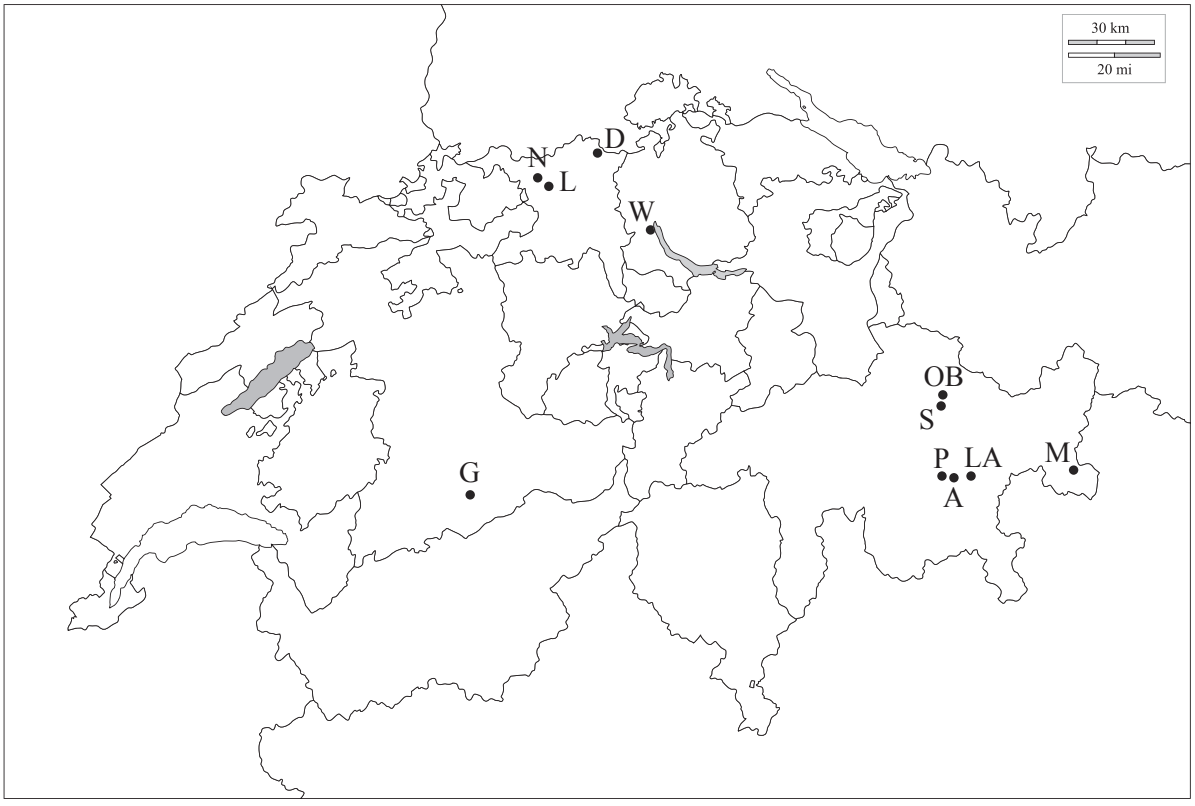

Fig. 2 Geographic locations of the study populations of *Gymandenia conopsea* and *G. odoratissima* in Switzerland. The populations are abbreviated as: Albula (A), Döttingen (D), Griesalp (G), Linn (L), La Punt (LA), Münstertal (M), Nätteberg (N), Obere Büschalp (OB), Preda (P), Schatzalp (S) and Wollishofen (W) (map modified from http://en.wikipedia.org/wiki/File:Switzerland_relief_location_map.jpg).

Reproductive Isolation

Floral isolation

Floral isolation was assessed through an experimental approach, where 40 plots were set up over four natural populations (Table S1). All populations contained sympatrically occurring *G. odoratissima* and *G. conopsea* in relatively high abundance. Each plot was composed of two individuals of each species, collected during the flowering period and placed individually in a 15mL Falcon™ tube (BD, Franklin Lakes, NJ, USA) containing water, and inserted into the ground. Within a plot, all four individuals were placed at an equidistance of approximately 20 cm, in a random order. Each plot was separated with an estimated distance of approximately two metres and at least half a metre from a naturally occurring neighbouring plant.

The pollinia of all the flowers of each individual (In both species, there are between 10–100 flowers on one individual) were colour-labelled with species-specific colours using histochemical stains (Peakall, 1989; Xu *et al.*, 2011); *G. odoratissima* pollinia were stained brilliant green, and *G. conopsea* pollinia were stained trypan blue. Pollinia staining was achieved by carefully injecting 1–2 μL of dye using a 10μL syringe under a light microscope (100× magnification, WILD-M4, Heerbrugg, Switzerland). The dyes do not interfere with the shape and cohesion of the pollinarium (Peakall, 1989) and do not affect the number of massulae deposited (Jersakova & Johnson, 2006).

Once per day, over 4 days, the number of pollinia removed and the deposition of stained pollen deposition on the floral stigma were identified through a hand lens (10× magnification, 20 mm Ruper field hand lens, Japan) and recorded. The floral isolation index was calculated using this formula:

$$\mathrm{RI}_{\mathrm{floral}} = 1 - (\text{number of interspecies transfers})/(\text{number of intraspecies transfers})$$

A RI value of 1 denotes complete reproductive isolation, whereas a value of 0 means no reproductive barrier is present.

Post-pollination prezygotic isolation: fruit production

Hand-pollinated crossing experiments were performed bidirectionally between *G. conopsea* and *G. odoratissima*. To exclude insect pollination, 46 individuals of each species were randomly marked in the field and covered with fine-mesh wire cages prior to flowering. Crosses were performed using a wooden toothpick to remove pollinia by touching the viscidium and placing the pollinia onto the stigma.

For interspecies crosses, a total of 63 flowers from 13 *G. conopsea* individuals and 74 flowers from 15 *G. odoratissima* individuals were outcrossed with pollen from the other species. For intraspecies crosses, a total of 215 flowers from 18 *G. conopsea* individuals and 88 flowers from 18 *G. odoratissima* individuals were outcrossed with pollen from another individual of the same species. To control for potential effects of inbreeding, an additional 5–10 flowers of each individual used in the inter- and intraspecies crosses were self-pollinated. Variation in the number of individuals used and the number of flowers pollinated depended on the number of individuals available in a population and the number of open flowers per individual. It was ensured that no more than half the number of flowers of each individual were pollinated, to prevent potentially negative effects, if any, of resource limitation on fruit set and seed development. In the case of orchids, the development of female gametophyte and subsequent enlargement of the ovary (i.e. fruit formation) is initiated when compatible pollen arrives into contact with the stigma (Zhang and O'Neill 1993). Through our hand pollination experiments, we are able to distinguish between post-mating prezygotic isolation, i.e. fruit formation, and post-mating post-zygotic isolation, i.e. seed viability. The mean number of fruits formed was counted for each set of treatment crosses. The post-pollination prezygotic isolation index of the crosses was estimated using the proportion of fruit set (based on Scopece *et al.*, 2007):

$$\mathrm{RI}_{\mathrm{post-pollination-prezygotic}} = 1 - (\text{average proportion of fruit set in interspecies crosses})/(\text{average proportion of fruit set in parental intraspecies crosses})$$

Post-zygotic isolation: seed viability and ploidy level

Ploidy level was analysed using flow cytometry (see supporting information). For seed viability, the proportion of seeds with developed embryos from the crosses was taken as an indication of seed viability. Seed germination was not measured in this study. Fruits from the inter- and intraspecific crosses were collected 3–4 weeks after hand pollination and stored in a −25 °C freezer. Each fruit capsule was dissected using a scalpel blade, and the seeds were released from the capsule onto a thin sheet of moist tissue. This was placed onto a petri dish marked with a 0.5 × 0.5 cm grid. The seeds were observed under a microscope (Olympus SZH-ILLD StereoZoom, Olympus Optical Co., LTD., Tokyo, Japan) with the illumination source from below. Squares within the grid were randomly selected, and all seeds within the squares were scored for the presence or absence of an embryo, that is viable or inviable seeds, respectively. No fewer than 100 seeds were scored per fruit to gain a representative ratio of seed viability. Through the estimation of the proportion of viable seeds for each type of reciprocal interspecies cross, the post-

zygotic index was deduced (based on Scopece *et al.*, 2007):

$$RI_{post-zygotic} = 1 - (\text{average proportion of viable seeds in interspecies crosses})/(\text{average proportion of viable seeds in parental intraspecies crosses})$$

Floral traits

Floral scent

Scent of *G. odoratissima* and *G. conopsea* inflorescences was collected from 20 individuals of each species during the day between 0800 and 1500 h. The inflorescence of each individual was completely enclosed in oven bags (Nalophan®; Kalle UK Ltd., Witham, UK) with approximate dimensions of 30 × 30 cm and sealed at the ends with twist close wires. A battery-operated pump (PAS-500 Personal Air Sampler, Spectrex Corp., Redwood City, CA, USA) attached to silicon rubber tubing connected to the inside of the bag was used to pull out air at a rate of 150 mL min^{-1} for 30 min. The air passed through a glass tube containing *ca.* 20 mg of Tenax® TA (80/100 mesh; Supelco, Bellefonte, PA. USA), in which scent compounds were adsorbed. The scent of the surrounding air was sampled as a control under the same collection parameters. After the collection time, glass tubes were sealed at both ends with PTFE tape (DuPont™ Teflon®) and stored in a −25 °C freezer until analysis using gas chromatography with mass selective detection (GC-MS).

The glass tubes were desorbed into a gas chromatograph (GC; Agilent 6890 N) using a Gerstel thermal desorption system (TDS3, Gerstel GmbH & Co. KG, Mühlheim, Germany) with cold injection system (CIS4; Gerstel). For thermal desorption, the TDS was heated from 30 °C (0.5 minute hold) to 240 °C (1 minute hold) at a rate of 60 °C min^{-1} five minutes. The CIS was set to −150 °C for the collection of eluting compounds from the TDS. The CIS was then heated up for injection from −150 °C (0.5 minute hold) to 150 °C at 16 °C s^{-1}, and from 150 °C to 250 °C (0.5 minute hold) at 12 °C s^{-1}. The oven temperature rose from 50 °C to 230 °C at 8 °C min^{-1} three minutes. Helium was used as the carrier gas at a flow rate of 1.9 mL min^{-1} through a HP-5MS column (0.25 μm internal diameter, 0.32 mm film thickness, 30 m length). Compound identification and quantification were attained with a mass selective detector (Agilent MSD 5975). The chromatogram data were analysed using the program Chemstation (G1701EA E.02.02 MSD Productivity ChemStation Software, Agilent Technologies, Germany), and the NIST spectral database within the program was implemented for preliminary identification of volatiles. The retention times and mass spectrograms of all floral volatiles were compared with those of synthetic reference compounds. For quantification, calibration curves for qualifier ions were established for all compounds through running the reference compounds in one or two different concentrations using the GC-MS system. To calculate the absolute amounts of floral volatiles, the peak areas of qualifier ions were converted into nanograms using the calibration curves. All compounds for all samples were manually rechecked and, if necessary, manually re-integrated. Compounds produced in only trace amounts were excluded by implementing a mean threshold of 0.5 ng L^{-1} of air sampled per inflorescence. In total, 31 compounds from the scent profile exceeded this threshold. Subsequently, the relative amount for each compound was calculated separately by dividing the absolute amount of a compound by the sum of the absolute amounts of all compounds collectively.

Floral colour

For each species, 12 individuals were randomly selected from which two flowers of each individual were sampled. One flower was removed from the top and one from the lower part of each inflorescence. The flower labellum colour was measured as percentage reflectance using a AvaSpec-2048 Fibre Optic Spectrometer (Avantes B.V., Eerbeek, the Netherlands) and a AvaLight-XE Xenon pulsed light source light (Avantes B.V.). The fibre optic probe (Avantes B.V.) was fixed at a constant distance and angle from the labellum using an enclosed fibre optic holder. The calibration was performed using a 98% reflective polytetrafluoroethylene (PTFE) white (reflects 98% of light from 350–1800 nm in wavelength) and black reference tile (Avantes B.V.) sequentially at the start of each measurement session. The reflectance spectra were displayed as the percentage of reflected light in relation to the white reference tile, taking into account the wavelengths between 300 and 700 nm. The output of the data collection software AvaSoft© 7.3 (AvaSoft-Basic, Avantes B.V.) were reflectance spectra each composed of 1206 percentage reflectance data points taken at 0.597 nm intervals. These were subsequently reduced to 10 nm intervals, with the mean percentage reflectance value at each reflectance interval derived from the two flowers of each individual.

Floral morphology

In total, 60 flowers from 20 individuals of *G. conopsea* and 40 flowers from 20 individuals of *G. odoratissima* were measured. Individuals were randomly selected, and the number of flowers per individual inflorescence was recorded. Two flowers (a higher and a lower flower on the inflorescence) per individual from *G. odoratissima* and three flowers (a higher, middle and lower flower on the inflorescence) per individual from *G. conopsea* were sampled and stored in individual glass vials containing 70% ethanol. In the laboratory, each individual flower was placed in a clear petri dish and thinly

immersed in two to three drops of 70% ethanol. The flowers were carefully spread out using a pair of fine tweezers to make all dimensions of the floral features fully visible. The flowers were displayed facing down such that the spur was entirely visible, revealing the exact location of the spur entrance, and then flattened into this position. Individual flower photos were taken using a digital SLR camera (Nikon D90 D-SLR; Nikon Corporation, Tokyo, Japan) fitted with a 105-mm F/2.8D lens (AF-S VR Micro-Nikkor; Nikon Corporation) and attached onto a fixed tripod.

For floral trait measurements, the image processing and analysis program ImageJ 1.47 (NIH Image, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/) was used. On each flower, 10 traits were measured to the nearest 0.001 mm with each measurement calibrated to a five-centimetre scale included in each photo (Fig. 3). The mean value for each trait was obtained by averaging each trait measurement between the two flowers of each *G. odoratissima* individual, and between the three flowers of each *G. conopsea* individual.

Data analysis

The computer software R (version 2.13.0, http://www.r-project.org/.) was used for all statistical analyses. To test for differences in individual floral scent compounds and morphological traits between the *G. conopsea* and *G. odoratissima*, one-way ANOVAs were conducted. Principal component analyses (PCAs) were conducted separately for the 31 scent compounds, 11 morphological traits and floral colour spectra to reduce the correlated variables into a few orthogonal variables (principal components, PCs). The PCAs were conducted using standardized trait values and varimax rotation. PCs with an eigenvalue greater than one were extracted. To examine species differences in the scent composition, morphology and colour spectra, the PC scores for each of the three traits were submitted to one-way ANOVAs. The seed set ratios and seed viability ratios of the outcrossed treatments were compared with the selfing treatments using paired *t*-tests to test for any effects of inbreeding. One-way ANOVAs with Tukey HSD *post hoc* tests were applied to assess differences in fruit set ratio and seed viability ratio between all crossing treatments.

Results

Phylogenetic analysis

Our phylogenetic analysis shows that Swiss *G. odoratissima* forms a sister group to Swiss *G. conopsea*, whereas Swiss *G. densiflora* forms a clade with *G. rhellicani* (Fig. 4).

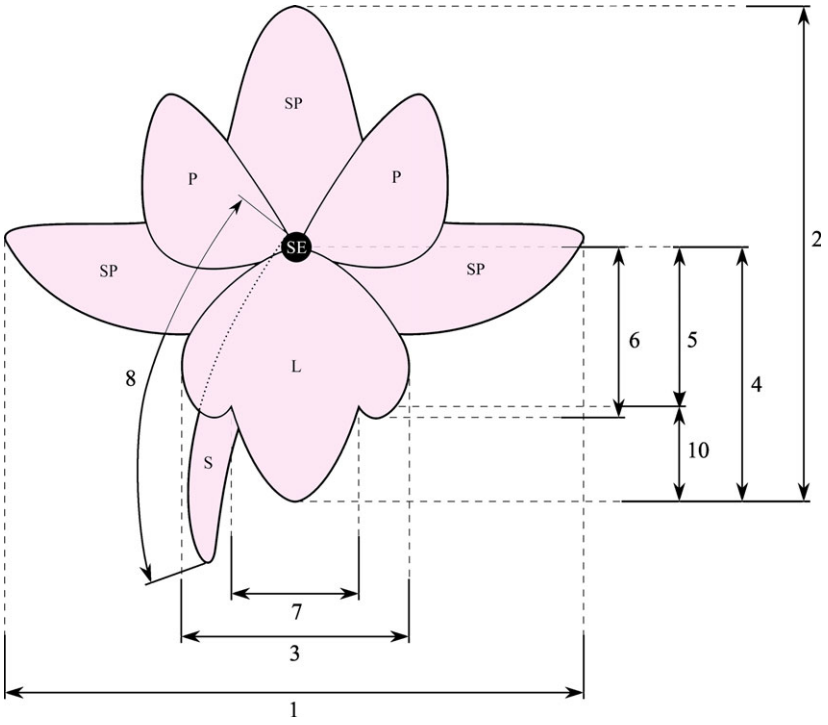

Fig. 3 A diagrammatic *Gymnadenia* flower with morphological traits 1–10 indicated. Morphological traits are flower width (1), flower height (2), labellum width (3), labellum height (4), spur entrance to height of interlobe (5), side-lobe length (6), interlobe distance (7), spur length (8), flower area (9), not shown, calculated by ImageJ through manually tracing the flower outline) and lobe length (10). Floral characters indicated are petals (P), sepals (SP), labellum (L), spur (S) and spur entrance (SE). The column of the flower is not shown.

Although our taxonomic sampling is incomplete, other phylogenetic analyses that include other European *Gymnadenia* species, further *G. odoratissima* and *G. conopsea* samples from different geographic locations, as well as the most widely distributed Asian species *G. orchidis*, are congruent with our analysis (Bateman *et al.*, 2003, 2006). Taken together, a sister–species relationship of *G. conopsea* and *G. odoratissima* is therefore likely.

Prepollination reproductive isolation

Floral isolation

A total of 162 stained pollination events occurred within all the plots. *G. conopsea* received 74 cases of stained pollination, whereas *G. odoratissima* received 88 cases. All pollination events occurred intraspecifically only, with no observed interspecies pollen transfers. Thus, the floral isolation index between *G. odoratissima* and *G. conopsea* equals one. As the percentage of autogamy and geitonogamy in *Gymnadenia* is approximately 50% (Gross & Schiestl, in press.), floral isolation is probably mediated half by autogamy/geitonogamy, and half by pollinator fidelity when moving between plant individuals.

Post-pollination isolation

Post-pollination prezygotic isolation: fruit set

A total of 117 interspecies, 293 intraspecies and 227 selfing hand pollinations were performed. Within the intraspecies crosses, there was no significant difference in fruit set ratio between the outcrossing and selfing treatments ($t_{51.01} = 0.58$, $P = 0.57$). This suggests that there is no effect of inbreeding depression in fruit set through selfing. Thus, for the interspecies crosses, the selfing fruit set ratio (performed to control for within individual differences) can be compared with the fruit set ratio of interspecific crosses. For all crossing treatments, there was no significant difference between the interspecific crosses and selfing fruit set ratio, and no significant difference between the (1) interspecific crosses bidirectionally and (2) the inter- and intraspecies crosses (Table S4). The $RI_{post\text{-}pollination\text{-}prezygotic}$ indices for the bidirectional crosses of the two species are shown in Table 2.

Post-pollination post-zygotic isolation: seed viability and ploidy level

Seeds were examined for the presence of embryos in a total of 48 fruits from interspecific crosses, 45 fruits from intraspecific crosses and 105 fruits from selfing hand pollinations. For the intraspecific crosses, the seed viability of fruits from selfing treatment was significantly lower than that of fruits from within-species outcrossing treatments for *G. conopsea* ($t_{33.28} = 4.79$, $P < 0.001$) and *G. odoratissima* ($t_{24.51} = 6.83$, $P < 0.001$), suggesting inbreeding depression. For the interspecific crosses, the viability ratio of the cross *G. odoratissima* (receiver) × *G. conopsea* (donor) was not significantly different from the viability ratio of the intraspecies crosses of both species (Table S5). However, the viability ratio of the cross in the other direction, that is *G. conopsea* (receiver) × *G. odoratissima* (donor), was not significantly different from the viability ratio of the *G. odoratissima* intraspecies crosses but was significantly lower than the viability ratio of the *G. conopsea* intraspecies crosses (Table S5; Table 1). There was no significant

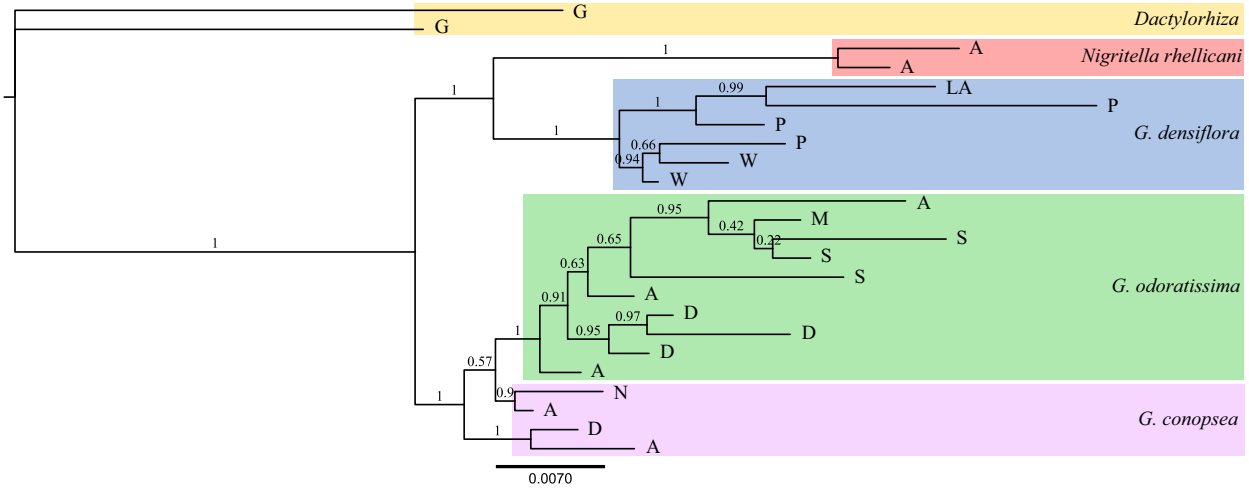

Fig. 4 Bayesian Inference consensus tree of five markers. The numbers at the nodes indicate Bayesian posterior probabilities. Branch length corresponds to the number of expected character changes per site along a branch (scale at the bottom). Coloured boxes indicate different species, and source populations are indicated as follows: Albula (A), Döttingen (D), Griesalp (G), La Punt (LA), Münstertal (M), Nätteberg (N), Preda (P), Schatzalp (S) and Wollishofen (W).

Table 1 Mean fruit set and seed viability percentage ratios from hand pollinated inter- and intraspecies crosses for *Gymnadenia odoratissima* and *G. conopsea*. The only significant difference ($P = 0.026$) was between the values marked with an asterisk; all *P*-values of statistical comparisons are given in Tables S4 and S5.

			Fruit set %			Viable seeds %		
Crossing	Pollen receiver ♀	Pollen donor ♂	*n*	Outcross (receiver × donor)	Selfing (within pollen receiver species)	*n*	Outcross (receiver × donor)	Selfing (within pollen receiver species)
Interspecies	*G. conopsea*	*G. odoratissima*	13	96.83	100.00	10	80.34*	75.81
	G. odoratissima	*G. conopsea*	11	85.19	89.74	5	91.84	57.43
Intraspecies	*G. conopsea*	*G. conopsea*	7	91.43	97.14	7	94.20*	72.12
	G. odoratissima	*G. odoratissima*	17	97.59	95.06	8	92.78	54.99

difference in the bidirectional comparison of the viability ratio in the cross between *G. conopsea* and *G. odoratissima* (Table S5). The $RI_{post\text{-}pollination\text{-}post\text{-}zygotic}$ indices for seed viability for the crossing treatments are shown in Table 2.

The overall RI indices between *G. conopsea* and *G. odoratissima* were high for floral isolation at 1, but low for both post-pollination prezygotic and post-pollination post-zygotic at 0.04 and 0.08, respectively. Among the here-investigated populations, individuals of both species were mainly diploid, with some polyploid individuals found within the populations (Table S6).

Floral trait differences

Floral scent

Several compounds differed significantly between the species (Fig. 5). The PCA yielded eight components (see SI Table S7 for compound loadings on each principle component, PC) within which three components (PC2, PC3 and PC5) were significantly different between the two species (SI Table S8, Figure S1). Five of the six known physiologically active compounds (Huber *et al.*, 2005) have significant loadings on these three PCs.

Floral colour

Three PCs were derived from the PCA, explaining 99.16% of the total variance in the data. The one-way ANOVA revealed significant differences only in PC1 in the relative reflectance of wavelengths between the two species ($F_{1,22} = 29.11$, $P < 0.001$; Fig. 6).

Floral morphology

All plant morphological traits were greater in *G. conopsea* compared with *G. odoratissima*, with the exception of the total number of flowers which was greater in *G. odoratissima* (Table 3). A highly significant difference between the two species is the spur length (Fig. 1), which is over three times longer in *G. conopsea* than in *G. odoratissima*. A single principal component (PC1) was identified through the principal component analysis, explaining 78.31% of the total variance. The PC1 loadings for almost all traits are high (approximately 0.9) apart from lobe length and number of flowers (SI Table S9). The one-way ANOVA on PC1 revealed a significant difference between the two species ($F_{35,\ 1} = 91.54$, $P < 0.001$).

Discussion

Empirical evaluation of the contribution of different barriers to gene flow and the maintenance of species integrity between relatively recently diverged species is important for understanding the speciation process. This present study provides estimates of reproductive isolation through the assessment of multiple reproductive barriers in two sympatrically occurring sister species of the genus *Gymnadenia*. We show that floral isolation is the main isolation mechanism between the two species, mediated by interspecies differences in floral scent, colour and morphology. We conclude that pollinator adaptation is likely to be the primary driver of speciation in these orchids.

Table 2 Post-pollination prezygotic and post-zygotic reproductive isolation index for bidirectional crosses between *Gymnadenia odoratissima* and *G. conopsea*. The reproductive isolation indices are shown for the maternal plant, paternal plant, as well as the subsequent total RI index.

		Post-pollination prezygotic RI index (fruit set)			Post-zygotic RI index (seed viability)		
Pollen receiver ♀	Pollen donor ♂	Maternal	Paternal	Total	Maternal	Paternal	Total
G. conopsea	*G. odoratissima*	−0.06	0.01	−0.03	0.15	0.13	0.14
G. odoratissima	*G. conopsea*	0.13	0.07	0.10	0.01	0.03	0.02

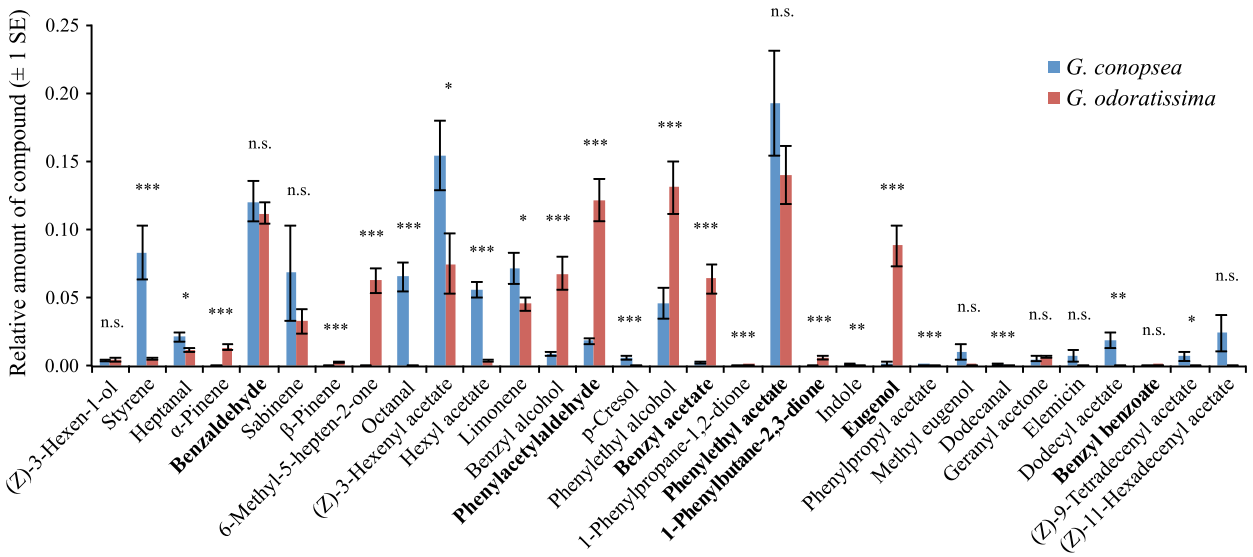

Fig. 5 The mean relative amount (± SE) of 31 floral scent compounds of *Gymandenia conopsea* (n = 20) and *G. odoratissima* (n = 20). Physiologically active compounds for pollinators of *Gymnadenia* are also marked in bold (based on Huber *et al.* (2005)). Results from the one-way ANOVA of the relative amounts of each compound compared between the two species are shown above the bars. Significant differences are shown as *: $P < 0.05$, **: $P < 0.001$, ***: $P < 0.0001$, n.s.: not significant.

Floral isolation in plant speciation

Floral isolation (or pollinator isolation) is an early acting reproductive barrier, unique to plants, mediated by the interaction of pollinators and floral traits (Grant, 1994; Johnson, 2006; Kay, 2006; Schiestl & Schlüter, 2009; Schiestl, 2012). Differences in floral morphology and floral signals are often integral to co-occurring

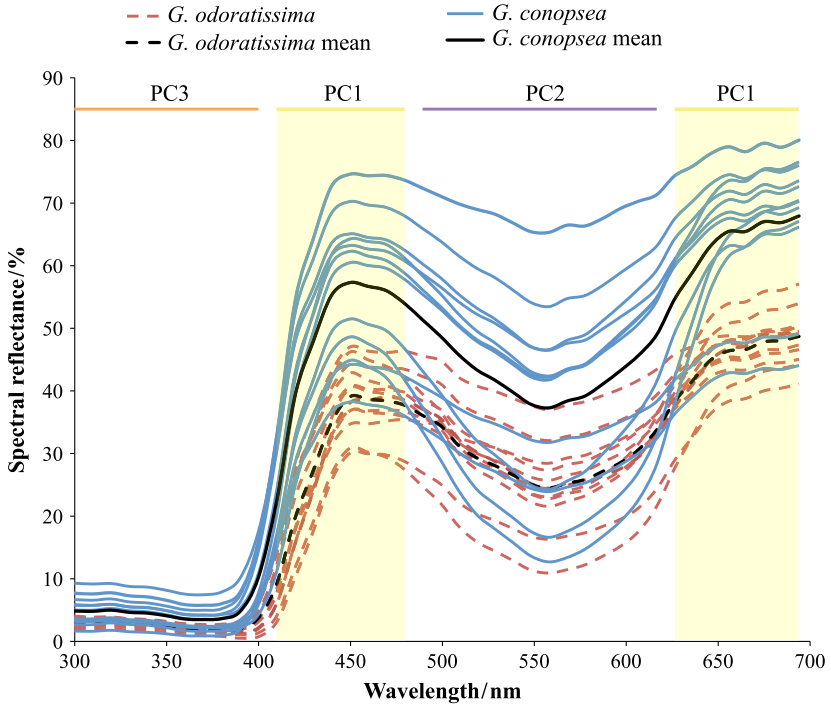

Fig. 6 The relative spectral reflectance (%) of *Gymnadenia odoratissima* individuals (red dashed lines; n = 12), *G. conopsea* individuals (blue solid lines; n = 12) and the species mean (black lines). The significant loadings of the wavelengths on PC1 (explaining 34.34% of the total variance in the data), PC2 (33.86%) and PC3 (30.95%) are marked.

Table 3 Mean (± SD) morphological trait values for flowers of *Gymnadenia conopsea* (n = 20) and *G. odoratissima* (n = 20). All units of absolute trait values are in mm, apart from 'flower area' (trait 9) which is measured in mm^2, and number of flowers. The numbers in brackets refer to the trait numbering in Fig. 3. Data for 'spur entrance to height of interlobe' (trait 5) was not included the analysis as it was used to derive 'lobe length' (trait 10) Results from one-way ANOVAS of morphological trait comparison between the two species are shown, with significant differences shown as *: $P < 0.05$, **: $P < 0.001$, ***: $P < 0.0001$.

	G. conopsea		*G. odoratissima*		
Trait	*n*	Mean ± SD	*n*	Mean ± SD	ANOVA $F_{38,1}$
Flower width (1)	19	12.65 ± 1.15	20	9.75 ± 0.81	82.86***
Flower height (2)	19	10.74 ± 1.22	20	8.35 ± 0.79	53.04***
Labellum width (3)	18	5.22 ± 0.77	20	3.73 ± 0.36	60.10***
Labellum height (4)	19	5.62 ± 0.64	20	4.10 ± 0.43	76.62***
Side-lobe length (6)	18	4.70 ± 0.74	19	3.04 ± 0.34	78.96***
Interlobe distance (7)	18	2.25 ± 0.26	19	1.75 ± 0.20	45.84***
Spur length (8)	19	17.07 ± 2.62	20	4.84 ± 0.58	415.83***
Flower area (9)	17	58.41 ± 11.97	20	36.04 ± 4.62	59.65***
Lobe length (10)	18	1.54 ± 0.34	20	1.24 ± 0.36	7.02*
Number of flowers	20	46.35 ± 14.94	20	67.60 ± 19.80	14.68**

plant species, suggesting floral isolation is widespread (Grant, 1994; Lowry *et al.*, 2008; Kay & Sargent, 2009; Schiestl & Schlüter, 2009). Floral isolation can be the primary basis of plant speciation, namely when plants adapt to different pollinators, and reproductive isolation is the sequential by-product of the utilization of different pollinator niches (Johnson, 2010; van der Niet & Johnson, 2012; Xu *et al.*, 2012). On the other hand, floral isolation can evolve secondarily to decrease mating between less compatible or differentially adapted genotypes. Notably, floral isolation can also evolve between nonrelated plant species, to optimize pollen export or pollination with compatible pollen in diverse plant communities. In this case, floral isolation is not involved in the speciation process, but evolves by reproductive character displacement (Armbruster *et al.*, 1994).

To gain more insight into the role of floral isolation in plant speciation, floral isolation must be studied in a phylogenetic context as well as in combination with other reproductive barriers. Our study shows that (a) the two *Gymnadenia* species are sister species, assuming that there was no extinction within the group and our taxonomic sampling was sufficient, and (b) floral isolation is the major reproductive barrier between the two species, consistent with the assumption that this prepollination barrier may have evolved first. Many studies have found that other isolation barriers, such as ecogeographical isolation or post-pollination barriers, act in concert with floral isolation (e.g. reviewed in Lowry *et al.*, 2008; Widmer *et al.*, 2009), rendering it problematic to assess which barrier evolved first and thus played the primary role in species divergence. Through a broad analysis of the patterns of reproductive isolation, Rieseberg *et al.* (2006) provided evidence that prepollination barriers often arise prior to the others. Despite that, early acting barriers such as pollinator and mating system isolation often evolve more rapidly and are stronger than later-acting, post-zygotic barriers (Rieseberg & Willis, 2007; and references within, Lowry *et al.*, 2008), examples of sister species with the relative strength of different reproductive barriers quantified remain scarce (Bradshaw & Schemske, 2003; Ramsey *et al.*, 2003). Our study suggests that the primary mechanism of speciation in these orchids is, therefore, likely to be adaptation to different suites of pollinators and/or different placement of pollinia on pollinators. In *G. odoratissima*, the important role of local adaptation to different pollinators has recently been demonstrated (Sun *et al.*, 2014). The formation of local 'pollinator races' are indeed thought to be an intermediate stage to full divergence via pollinator adaptation (Grant, 1971; Johnson, 2006). In our study, we have not analysed late-acting post-zygotic reproductive barriers such as hybrid viability and hybrid reproductive success, due to the constraints inherent in growing orchids from seeds. Late-acting barriers are, however, of little significance for the total reproductive isolation if early acting barriers such as floral isolation are strong.

Floral isolation and floral traits

Floral isolation can be mediated by the attraction of different pollinators or placement of pollen on different anatomical parts of shared pollinators (Grant, 1994). The basis for different pollinator attraction can be either in the form of an optimal fit between the pollinator and flower, allowing an efficient exploitation of floral rewards, or the production of floral signals that result in specific pollinator attraction mediated by innate preferences (Schiestl & Schlüter, 2009). In the *Gymnadenia* system, floral isolation is likely to be a product of differences in both flower morphology and floral signals, as these traits differed strongly between the two species. This combination of traits in contribution towards floral isolation is common, for example in the food-rewarding

genera *Mimulus* (Ramsey *et al.*, 2003), *Aquilegia* (Fulton & Hodges, 1999; Hodges *et al.*, 2002), as well as in *Petunia* (Dell'Olivo *et al.*, 2011). In *Gymnadenia*, the strong discrepancies in floral spur length suggest that the species may have adapted to pollinators with different proboscis lengths. Systems where floral signals alone mediate floral isolation are less common and are exemplified in flowers that mimic sexual partners of their pollinators or oviposition sites and exploit the 'hard-wired' responses of pollinators to signals that are normally associated with a model item (i.e. the mating partner, oviposition site, etc.; Schiestl & Schlüter, 2009; Urru *et al.*, 2011). In some sexually deceptive orchids, floral isolation is mediated solely by the production of different floral scent bouquets (Xu *et al.*, 2011) or the production of few different scent compounds (Peakall *et al.*, 2010; Peakall & Whitehead, 2014).

In the two *Gymnadenia* species, two scenarios are possible for the evolution of the combination of differences in both traits. In the first, and most plausible scenario, the spur length difference evolves initially, mediated by disruptive selection on spur length (Rymer *et al.*, 2010). Such disruptive selection could be driven by density-dependent selection for the utilization of pollinators with different proboscis lengths. In the second scenario, changes in floral signals occur prior to morphological changes. This is, however, only possible if groups of pollinators show strong and consistently divergent preferences for floral signals. As signals in food-rewarding pollination systems are often learnt by pollinators, there is usually great flexibility in terms of which signals pollinators are attracted to (Schiestl & Johnson, 2013). Thus, consistent disruptive selection on floral signals is unlikely to be a primary mechanism of divergence in these systems. Rather, different signals can evolve secondarily in plants that utilize different pollinators as a learning cue for pollinators in finding appropriate food sources and ensuring efficient and reliable intraspecific pollen transfer.

In conclusion, we show strong floral isolation in a pair of co-occurring orchid sister species, making differential pollinator adaptation the most likely mechanism for their speciation. Future studies should quantify pollinator-mediated selection in different populations as well as genetic variability in floral traits to increase our understanding of the mechanisms of divergence in these orchids and our knowledge of angiosperm speciation in general.

Acknowledgments

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Geographical details of the study populations and the year of sampling of *Gymnadenia conopsea* (Gc), *G. odoratissima* (Go), *G. densiflora* (Gd), *Nigritella rhellicani* (Nr), *Dactylorhiza majalis* (Dmj) and D. *maculata* (Dmc) for the assessment of reproductive isolation barriers and floral traits, as well as for phylogenetic analysis.

Table S2 Oligonucleotides used to amplify and sequence the five markers in this study, and the PCR programmes for amplification. References to published primers are listed.

Table S3 The best-fitting models of nucleotide substitution used in the Bayesian analysis for each marker under investigation.

Table S4 *P*-values of one-way ANOVAS with Tukey HSD *post hoc* tests comparing the fruit set ratios between all crossing treatments.

Table S5 *P*-values of one-way ANOVAS with Tukey HSD *post hoc* tests comparing the seed viability ratios between all crossing treatments.

Table S6 The number of diploid (2×), triploid (3×), and tetraploid (4×) plants of the two species *Gymnadenia conopsea* and *G. odoratissima* in eight sympatric natural populations.

Table S7 Rotated PCs derived from a PCA of all 31 floral scent compounds in *G. conopsea* and *G. odoratissima*.

Table S8 ANOVA comparison between species on PCs 1–8 derived from a PCA on all 31 floral scent compounds in *G. conopsea* and *G. odoratissima*.

Table S9 Eigenvector scores of floral traits 1–11 (for trait identification see Fig. 2) in PCA axis 1.

Figure S1 Floral scent bouquet analysis of the scent compound composition between *G. conopsea* (blue circles, $n = 20$) and *G. odoratissima* (red triangles, $n = 20$).

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Supporting Information

Expanded materials and methods

Table S1. Geographical details of the study populations and the year of sampling of *Gymnadenia conopsea* (Gc), *G. odoratissima* (Go), *G. densiflora* (Gd), *Nigritella rhellicani* (Nr)*, Dactylorhiza majalis* (Dmj) and *D. maculata* (Dmc) for the assessment of reproductive isolation barriers and floral traits, as well as for phylogenetic analysis.

Population		Reproductive isolation barriers			Floral traits			Phylogeny
Name	GPS coordinates	Pre-pollination (floral isolation)	Post-pollination, pre-zygotic (fruit set)	Post-zygotic (seed viability)	Scent	Colour	Morphology	
Döttingen	N: 47˚34’28” E: 8˚16’21” 500 m a.s.l.	2010 (Gc, Go)	2011 and 2012 (Gc, Go)	2011 and 2012 (Gc, Go)	2011 (Gc, Go)	2012 (Gc, Go)	2010 (Gc, Go)	2010 (Gc, Go)
Linn	N: 47˚28’37” E: 8˚7’1” 510 m a.s.l.						2010 (Go)	
Schatzalp	N: 46˚48’21” E: 09˚49’32” 1780 m a.s.l.	2012 (Gc, Go)						2010 (Go)
Obere Büschalp	N: 46˚48’36“ E: 09˚49’28“ 1940 m a.s.l.		2011 (Gc, Go)	2011 (Gc, Go)				
Preda	N: 46˚35’24” E: 09˚46’37” 1810 m a.s.l.	2010 (Gc, Go)						2010 (Gd)
Münstertal	N: 46˚47’48” E: 10˚19’08” 1810 m a.s.l.	2010 (Gc, Go)					2010 (Go)	2010 (Go)
Albula	N: 46°34'54” E: 09°48'50” 2250 m a.s.l.							2010 (Nr, Go, Gc)
Griesalp	N: 46°54'37” E: 7˚74’59” 1560 m a.s.l.							2010 (Dmc, Dmj)
La Punt	N: 46°57'24” E: 09°93'99” 1810 m a.s.l.							2010 (Gd)

Nätteberg	N: 47˚29’42” E: 8˚05’47” 490 m.a.s.l.							2010 (Gc)
Wollishofen	N: 47°33'18" E: 8°53'02" 467 m a.s.l.							2010 (Gd)

Phylogenetic analyses

Plant material from 24 individuals was collected in nine *Gymnadenia* populations in Switzerland during their flowering time in 2010. The genus *Dactylorhiza* was chosen as an outgroup as it is known to be the sister species of *Gymnadenia* (Bateman et al. 2003; Bateman et al. 2006). Leaf tissue samples were cut from each individual, placed in separate plastic bags, and stored at -80 ºC until analysis. DNA was extracted using the CTAB procedure following the slightly modified protocol of Doyle and Doyle (1990) and the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). In total, DNA was amplified from five nuclear markers, namely internal transcribed spacers 1 and 2 (ITS1, ITS2), anthocyanin O-methyltransferase (*AOMT*), chalcone synthase (*CHS*) and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*). All primers are listed in Table S1. Sanger sequencing was carried out using BigDye 3.1 on an ABI PRISM 3130xl sequencing system (Applied Biosystems, Foster City, CA 94404 USA) using the manufacturer’s protocols. All sequences obtained in this study are available from genbank under accession numbers X-Y.

Sequences were aligned using MUSCLE (Edgar 2004) with manual adjustments in BioEdit 7.0.9.0 (Hall 1999), and used for phylogenetic analysis by Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Appropriate nucleotide substitution models (Table S2) were inferred using MrModeltest 2.3 (Nylander et al. 2004) and the Akaike information criterion. BI in MrBayes (2 runs, 4 MCMC chains per run) was automatically stopped when the standard deviation of split frequencies fell below 0.01 (633 000 generations) and accepted when the potential scale reduction factor (PSRF) (Brooks and Gelman 1998) for all parameters was 1 ± 0.01, indicating

convergence of both runs. Trees were sampled every 1000 generations, with the first 100 samples discarded as burn-in.

Table S2. Oligonucleotides used to amplify and sequence the five markers in this study, and the PCR programmes for amplification. References to published primers are listed.

Marker	Primer	Sequence 5' → 3'	PCR cycling program	Reference
ITS1	a	TCGTAACAAGGTTTCCGTAGG	96°-20s,40x(94°-20s,54°-20s,72°-60s),72°-10min	Leskinen *et al.* (1997)
	b	GCTGCGTTCTTCATCGWTG		Leskinen *et al.* (1997)
ITS2	c	CAWCGATGAAGAACGCAGC	96°-20s,40x(94°-20s,54°-20s,72°-60s),72°-10min	Leskinen *et al.* (1997)
	d	TTCCTTCCGCTTATTGATATGC		Leskinen *et al.* (1997)
CHS	CHS-SF	TACTTCAGAATAACCGGGAGCGAA	96°-20s,40x(94°-20s,50°-20s,72°-80s),72°-10min	Inda *et al.* (2010)
	CHS-R	AGCACGCABGCGCTBGACATGTT		Inda *et al.* (2010)
	CHS-F2	GCCGTTCATCTTCCATCATC		This study
	CHS-R2	TGAACGCCTCCATAAGACTC		This study
AOMT	AOMT-F	GCAAAGCGAAGCTCTTCATC	96°-20s,40x(94°-20s,50°-20s,72°-60s),72°-10min	Gupta unpublished
	AOMT-R1	TATTGATGAATGCGGGAACA		This study
	AOMT-R2	TCTCTTGCCGTTCATGACCT		This study
G3PDH	OsG3PDH-1F	ATGTTCAAGTATGACACTGTGCATGG	96°-20s,40x(94°-20s,46°-20s,72°-60s),72°-10min	Schlüter *et al.* (2011)
	OsG3PDH-2R	GTCGGCACACGGAAAGACATACCAGTCAA		Schlüter *et al.* (2011)
	GcG3PDH-F2	GTGTTAATGAGCACGAGTACA		This study

Table S3: The best-fitting models of nucleotide substitution used in the Bayesian analysis for each marker under investigation. The length of each marker, its position within the final alignment and the number of parsimony informative characters are indicated.

Marker	Nucleotide substitution model	Position in alignment	Length (bp)	Parsimony informative characters (% of total length)
ITS1	SYM+Γ	1-256	256	15 (5.6)
ITS2	K80+I+ Γ	257-683	427	20 (4.7)
CHS	HKY+ Γ	684-1378	695	63 (9)
AOMT	GTR+ Γ	1379-2247	869	35 (4)
G3PDH	HKY+I+ Γ	2248-3389	1‘142	108 (9.4)

Ploidy analysis by flow cytometry

From 14-25 plants of *G. odoratissima* and from 16-49 plants of *G. conopsea* per populations, two to six pollinaria were collected in 1.5 ml-Eppendorf® tubes and stored in a fridge at 4 °C. To analyse the relative ploidy level of each individual, we used a Cell

Lab QuantaTM SC-MPL flow cytometer (Beckman Coulter, Fullerton, Canada) equipped with a mercury arc lamp. Sample preparation and analysis followed a two-step protocol (Doležel, Greilhuber et al. 2007). All two to six pollinaria were chopped and mashed together with approximately 25 mm^2 leaf material of an internal standard (*Phaseolus coccineus*; 2n, 1C = 1.01 ± 0.4 pg (Bennett and Leitch 2005)) with a sharp razor blade in 1 ml ice-cold Baranyi's solution (0.1 M citric acid, 0.5% Triton X-100 (Baranyi and Greilhuber 1995)). The suspension was filtered through a 30 µm CellTrics® disposable filter (Partec GmbH, Münster, Germany) and the filtrate was centrifuged (5 min, 380 × g, room temperature) using a Sorvall® RMC 14 centrifuge (Kendro Revco Lindberg Heraeus Sorvall, Asheville, NC). The supernatant was removed and nuclei were resuspended in 40 µl ice-cold Baranyi's solution. In the Cell Lab QuantaTM SC-MPL flow cytometer, 160 µl Otto II solution (0.4 M Na_2HPO_4) containing 4 µg ml^{-1} DAPI (4′, 6-diamidino-2-phenylindole) were added and relative fluorescence intensity was recorded. Analysis was stopped when a total count of 8000 particles was reached or after 5 min. To determine relative ploidy levels, the ratio between the median of first pollinia peaks and the median of first peak of the internal standard (P:IS ratio) was calculated. As an earlier study showed that the lowest ploidy level in *Gymnadenia* seems to be diploid (Trávníček, Jersáková et al. 2012), we assumed that the pollinaria with the lowest relative ploidy were haploid produced by a diploid plants. We found three distinct relative ploidy levels, which represent diploid (2x; lowest relative ploidy), triploid (3x; medium relative ploidy), and tetraploid (4x; highest relative ploidy) plants.

Results

Interspecific crosses

Table S4. P-values of one-way ANOVAs with Tukey HSD post-hoc tests comparing the fruit set ratios between all crossing treatments. Pollen receiver plant: ♀, pollen donor plant: ♂, *Gymnadenia conopsea*: Gc, *G. odoratissima*: Go; Intraspecies refers to outcrosses.

		Interspecies		Intraspecies	
		Gc(♀) x Go(♂)	Go(♀) x Gc(♂)	Gc	Go
Interspecies	Gc(♀) x Go(♂)				
	Go(♀) x Gc(♂)	0.911			
Intraspecies	Gc	0.988	1.000		
	Go	1.000	0.841	0.967	

Table S5. P-values of one-way ANOVAs with Tukey HSD post-hoc tests comparing the seed viability ratios between all crossing treatments. Seed viability ratios that are significantly different between a crossing treatment pair are highlighted in bold. Pollen receiver plant: ♀, pollen donor plant: ♂, *Gymnadenia conopsea*: Gc, *G. odoratissima*: Go; Intraspecies refers to outcrosses.

		Interspecies		Intraspecies	
		Gc(♀) x Go(♂)	Go(♀) x Gc(♂)	Gc	Go
Interspecies	Gc(♀) x Go(♂)				
	Go(♀) x Gc(♂)	0.320			
Intraspecies	Gc	**0.026**	0.999		
	Go	0.090	0.999	1.000	

Ploidy level

Table S6. The number of diploid (2x), triploid (3x), and tetraploid (4x) plants of the two species *Gymnadenia conopsea* and *G. odoratissima* in eight sympatric natural populations.

Population	Sampling year	*G. conopsea*			*G. odoratissima*		
		# 2x	# 3x	# 4x	# 2x	# 3x	# 4x
Döttingen	2010	49	0	0	15	0	0
Remigen	2010	49	0	0	14	0	0
Linn	2010				15	0	0
Nätteberg	2010	25	0	0			
Schatzalp	2010				15	1	0
Albulapass	2010	16	0	0	23	1	1
Münstertal	2010				15	0	0
Münstertal	2011				20	0	0
Cinuos-chel - Brail	2011	17	0	2			

Table S7. Rotated PCs derived from a PCA of all 31 floral scent compounds in *G. conopsea* and *G. odoratissima*. In total, eight components were extracted with loadings on each component shown. For each scent compound the PC with the greatest loading is highlighted in bold. Compounds that are physiologically active for pollinators of *Gymnadenia* based on Huber et al. (2005) are also marked in bold.

	Component (% of variance)							
	PC1 (19.43 %)	PC2 (14.74 %)	PC3 (9.57 %)	PC4 (9.34 %)	PC5 (8.77 %)	PC6 (8.02 %)	PC7 (6.90 %)	PC8 (4.57 %)
(Z)-3-Hexen-1-ol	-0.13	0.32	-0.26	**0.71**	-0.13	-0.11	-0.10	-0.09
Styrene	-0.17	-0.31	**-0.48**	-0.10	-0.40	0.18	-0.29	0.00
Heptanal	0.25	0.01	-0.32	0.19	-0.04	**0.80**	0.15	0.02
α-Pinene	-0.17	**0.81**	0.31	-0.16	0.08	-0.03	0.01	-0.03
Benzaldehyde	-0.07	0.07	0.00	-0.18	-0.16	**0.76**	-0.29	0.25
Sabinene	-0.09	-0.01	-0.09	-0.08	-0.20	-0.26	**0.89**	0.13
β-Pinene	-0.13	**0.78**	0.20	-0.07	-0.06	-0.01	0.07	-0.04
6-Methyl-5-hepten-2-one	-0.15	**0.86**	0.03	0.01	0.31	-0.04	-0.01	0.03

Octanal	0.41	-0.44	-0.18	0.27	-0.15	**0.59**	0.20	-0.14
(Z)-3-Hexenyl acetate	0.00	-0.08	-0.22	**0.89**	-0.21	-0.06	-0.07	0.12
Hexyl acetate	0.25	**-0.52**	-0.39	0.28	-0.28	0.40	0.00	-0.10
Limonene	0.19	0.06	-0.26	-0.04	-0.15	0.23	**0.85**	-0.19
Benzyl alcohol	-0.20	0.16	0.10	-0.18	**0.71**	-0.13	-0.12	0.27
Phenylacetaldehyde	-0.19	0.18	**0.81**	-0.21	0.15	-0.15	-0.17	-0.06
p-Cresol	0.20	-0.41	-0.03	**0.69**	-0.09	0.22	0.11	0.01
Phenylethyl alcohol	-0.24	0.21	-0.07	-0.21	**0.74**	-0.16	-0.17	-0.13
Benzyl acetate	-0.17	0.34	**0.74**	-0.22	-0.11	-0.06	-0.14	0.06
1-Phenylpropane-1,2-dione	-0.18	**0.83**	0.24	-0.09	0.21	0.03	-0.01	0.06
Phenylethylacetate	-0.26	-0.30	0.00	-0.31	-0.31	-0.27	-0.43	**-0.43**
1-Phenylbutane-2,3-dione	-0.13	0.18	**0.82**	-0.17	0.13	-0.09	-0.11	-0.02
Indole	**0.88**	-0.17	-0.08	0.10	-0.09	0.13	0.12	-0.04
Eugenol	-0.15	0.23	0.24	-0.14	**0.69**	-0.16	-0.07	0.29
Phenylpropyl acetate	0.09	-0.44	-0.13	**0.70**	-0.16	0.16	0.01	-0.07
Methyl eugenol	0.57	-0.09	-0.05	0.00	-0.05	0.29	-0.02	**0.62**
Dodecanal	**0.77**	-0.26	-0.17	0.13	-0.12	0.34	0.16	-0.19
Geranyl acetone	**0.55**	0.55	-0.16	0.13	0.15	0.21	0.15	-0.29
Elemicin	**0.79**	-0.09	-0.05	0.00	-0.09	0.15	-0.04	0.51
Dodecyl acetate	**0.92**	-0.17	-0.13	0.02	-0.13	0.17	0.02	0.20
Benzyl benzoate	0.00	0.01	0.05	-0.08	**0.61**	0.07	-0.03	-0.22
(Z)-9-Tetradecenyl acetate	**0.96**	-0.11	-0.07	0.00	-0.09	-0.05	0.00	0.10
(Z)-11-Hexadecenyl acetate	**0.91**	-0.07	-0.07	-0.03	-0.08	-0.17	-0.01	-0.09

Table S8. ANOVA comparison between species on PCs 1-8 derived from a PCA on all 31 floral scent compounds in *G. conopsea* and *G. odoratissima*. PCs that are significantly different between the two species are highlighted in bold.

	$F_{38,1}$	p
PC1	2.10	0.16
PC2	**27.81**	**<0.001**
PC3	**12.28**	**0.001**
PC4	0.87	0.36
PC5	**7.91**	**0.01**
PC6	1.26	0.27
PC7	0.43	0.51
PC8	0.27	0.60

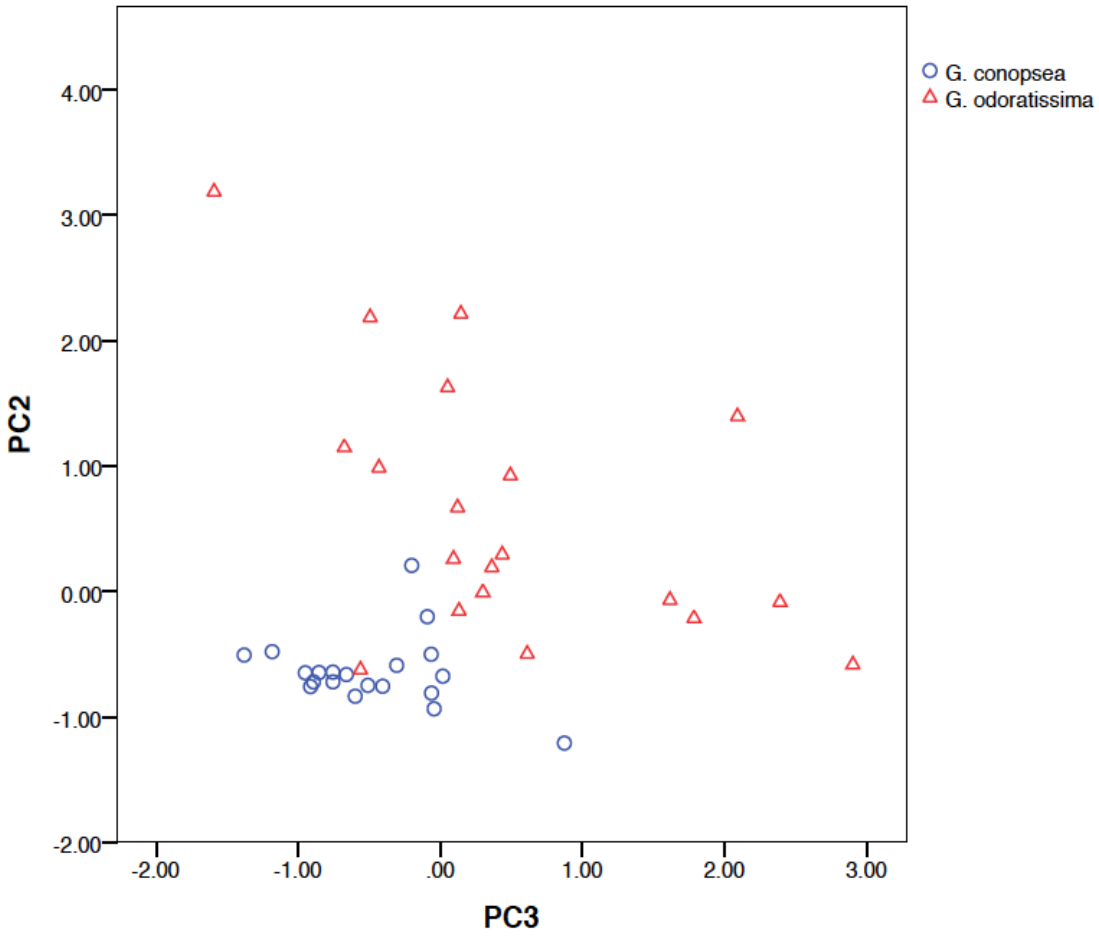

Figure S1. Floral scent bouquet analysis of the scent compound composition between *G. conopsea* (blue circles, n = 20) and *G. odoratissima* (red triangles, n = 20). PCA yielded two main components, PC2 (14.74 %) and PC3 (9.57 %), which were significantly different between the two species.

Table S9. Eigenvector scores of floral traits 1-11 (for trait identification see Fig. 2) in PCA axis 1. Values are ranked in order of absolute magnitude. The high eigenvector scores for each PCA axis are indicated in bold.

	PC1 (78.31 %)
Flower area	**0.975**
Flower width	**0.969**
Flower height	**0.935**
Labellum height	**0.981**
Se-side lobe	**0.956**
Labellum width	**0.934**
Labellum	**0.849**
Spur length	**0.880**
Interlobe distance	**0.979**
Lobe length	0.574
Number of flowers	-0.567

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Curriculum Vitae

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EDUCATION

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RESEARCH AND PROJECT INTERESTS

Ph.D. project: Pollinator-mediated evolution
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M.Res. projects:

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TEACHING EXPERIENCE

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CONFERENCES

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WORKSHOPS

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AWARDS & GRANTS

The Society for the Study Evolution (SSE) International Travel Award 2011 – 500 USD
Georges and Antoine Claraz-Schenkung Fund 2012 – 2019 CHF
Georges and Antoine Claraz-Schenkung Fund 2013 – 1000 CHF

PUBLICATIONS

Sun, M., Gross, K. & Schiestl, F.P. (2014) Floral adaptation to local pollinator guilds in a terrestrial orchid. *Annals of Botany*, 113 (2): 289-300

Sun, M., Schlüter, P.M., Gross, K. & Schiestl, F.P. (2015) Floral isolation is the major reproductive barrier between a pair of rewarding orchid sister species. *Journal of Evolutionary Biology*, 28 (1): 117-129

Gross, K., **Sun, M.**, & Schiestl, F.P. (in review) Region-specific selection on floral scent in a terrestrial orchid. *The American Naturalist*.

In preparation:

Sun, M., Gross, K. & Schiestl, F.P. (in prep.) Regional differences in selection on floral morphology.

Sun, M. & Schiestl, F.P. (in prep.) The role of scent in reproductive isolation in the deceptive orchid genus *Ophrys*.